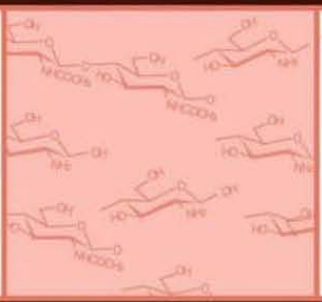
Chitin, Chitosan, Oligosaccharides and Their Derivatives

Biological Activities and Applications



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Se-Kwon Kim

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Contents

Preface

With better appreciation of biopolymers derived from marine organisms, there has been increased interest in their biomedical and industrial applications. Some such important molecules are chitin, chitosan, oligosaccharides, and their derivatives, which have attracted significant interest in view of their broad range of applications, including in the biomedical, agricultural, food science, and technological fields, and in various industries. Chitin is a naturally abundant mucopolysaccharide, and is the second-most abundant natural biopolymer after cellulose. Nature produces approximately 10^{11} tons of chitin annually worldwide as a by-product, and industrial use has been estimated at 10,000 tons annually. Chitosans are water-insoluble polymers, formed by the deacetylation of chitins, and have important biological properties. In order to exploit the potential of these molecules more fully and to increase their applications in diverse scientific areas, researchers transformed these complex substances into low-molecular-weight oligosaccharides known as chitooligosaccharides (COSs). As in the case of amino acids, which are building blocks of proteins; less than 100 units of glucosamine get connected to form COSs, which have low viscosity and relatively small molecular sizes [<1–18 kDa, i.e., 100 × 180 (MW of the glucosamine)], which in turn make them water soluble and readily absorbable in *in vivo* systems. A wide range of sources and technological approaches for these biologically useful biopolymers have been identified, but the significance of their diversity and the applicability of the different forms of these remarkable substances have yet to be determined. A wealth of knowledge and diversity is continuously being added to the oceans, which are the key sources for these natural substances as compared to terrestrial resources.

This book, *Chitin*, *Chitosan*, *Oligosaccharides*, *and Their Derivatives: Biological Activities and Applications*, covers the key aspects of chitin, chitosan, oligosaccharides, and their derivatives, namely, their properties, sources, production, and applications in the biological, biomedical, industrial, and agricultural fields. Part I provides an overview of the sources and production of chitin and chitosan derivatives; Part II describes their physical and chemical aspects; Part III discusses their structural modifications for biomedical applications; Part IV deals with their biological activities, in particular, antimicrobial, anti-inflammatory, antioxidant, antihypertensive, anticancer, and antidiabetic activities; Part V describes their biomedical applications, including their possible applications as drug, vaccine, and gene carriers; Part VI discusses their industrial applications; and, finally, Part VII covers their agricultural applications. The chapters in each part are a good collection of comprehensive research on these polymers carried out by proficient scientists from around the world. In addition, the preparation methodologies for these polymers have also been well depicted by various contributors in their respective chapters. I am quite certain that the findings and latest information presented in this book will be helpful for upcoming researchers to establish phenomenal research from an intersection of multiple research areas.

I am grateful to all the chapter authors who have provided the state-of-the art contributions in the field of chitin/chitosan; their relentless effort was the result of scientific attitude, drawn from the past history in this field. I also thank the staff of Taylor & Francis Group and CRC Press for their continual support, which was essential for the successful completion of this book. I hope that the fundamental ideas presented in this book serve as potential research and development material for the benefit of humankind.

Se-Kwon Kim

Editor

Se-Kwon Kim is a professor in the Department of Chemistry and the director of the Marine Bioprocess Research Center (MBPRC) at the Pukyong National University, Busan, South Korea. He received his MSc and PhD from the same university and conducted his postdoctoral study at the Laboratory of Marine Biochemical Engineering at the University of Illinois, Urbana-Champaign, Illinois (1988–1989). Later, he became a visiting scientist at the Memorial University of Newfoundland in Canada (1999–2000). Dr. Kim served as president of the Korean Society of Chitin and Chitosan (1986–1990) and the Korean Society of Marine Biotechnology (2006–2007). He won the best paper award from the American Oil Chemists' Society in 2002. Prof. Kim was also the chairman for the 7th Asia-Pacific Chitin and Chitosan Symposium, which was held in South Korea in 2006. He is one of the board members of the International Society of Marine Biotechnology (IMB) and the International Society for Nutraceuticals and Functional Foods (ISNFF). His major research interests are the investigation and development of bioactive substances derived from chitin, chitosan, and their derivatives, and their application in marine bioprocessing and mass-production technologies for the marine bio-industry. Furthermore, he extended his research fields to include the development of bioactive materials from marine organisms for applications in oriental medicine, cosmeceuticals, and nutraceuticals. To date, he has authored over 400 research papers and holds 65 patents.

About the Book

Marine animals and plants have lots of pharmaceutical potential as they possess biologically important molecules as compared with terrestrial organisms. Humans have studied the mechanism of producing natural molecules and have taken advantage of these molecules by isolating them and using them in various biotechnological, medical, as well as industrial applications. The basic concept of this book is to draw attention to biopolymers such as chitin, chitosan, oligosaccharides, and their derivatives, which have the most therapeutic value. Their forms, functions, and applications in various fields like food science technology, biotechnology, medicine, and industries are also treated in detail. Experimentally, it was seen that these substances were very active against various infectious, inflammatory, oxidative, as well as carcinogenic factors, and, hence, could serve as the basis for developing functional foods or drugs.

The first three parts in the book cover the sources, physical and chemical properties, and structural modifications of chitin, chitosan, oligosaccharides, and their derivatives, including the isolation and production of these molecules from different organisms. The various techniques and technologies for chitin/chitosan studies have been well explained in a few of the chapters for a better understanding of the potential of these molecules. The remaining four parts deal with the different activities and applications of these molecules in biotechnology, medicine, agriculture, and industrial applications.

This entire book was edited by leading experts in the field of natural biomaterials, and will be a valuable reference source for researchers working in this field.

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Part I

The Sources and Production of Chitin and Chitosan Derivatives

1 Chitin and Chitosan from Terrestrial Organisms

Nitar Nwe, Tetsuya Furuike, and Hiroshi Tamura

CONTENTS

1.1 INTRODUCTION

Chitin is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine units linked with β-(1-4) glycosidic bond, where *N*-acetyl-D-glucosamine units are predominant in the polymeric chain. The deacetylated form of chitin refers to chitosan. Chitin and chitosan can be found as supporting materials in many aquatic organisms, terrestrial organisms, and some microorganisms (Tokura and Tamura 2007). Nowadays, commercially, chitins and chitosans are produced from biowastes obtained from aquatic organisms. The production of chitin and chitosan from biowastes of aquatic organisms in industrial scale appear in inconsistent physicochemical characteristics of products because of seasonal and variable supply of raw materials as well as variability and difficulties of process conditions (Crestini et al. 1996, Nwe and Stevens 2008). To overcome these problems, terrestrial organisms like insects, terrestrial crustaceans, and mushrooms are considered as alternative sources for the production of chitin and chitosan.

Among the terrestrial organisms, silkworms, honeybees, and mushrooms have been cultured in industrial scale to produce valuable products for human use in Southeast Asia, United States, and Europe (Haga 2003, Nemtsev et al. 2004, Wu et al. 2004, Paulino et al. 2006, Yen and Mau 2007b, Synytsya et al. 2009). In the silk industry, a massive amount of waste materials, several thousand tons per year, remains at the end of the process (Paulino et al. 2006). In the dead silkworm larvae bodies, 680 g/1000 bodies is composed of 24% of cuticle (Haga 2003). Also, 6,000–10,000 tons per year from honeybees can be obtained after the process of honey harvesting in the rural economy of the Russian Federation (Nemtsev et al. 2004). These materials have been proposed for the large-scale production of chitin and chitosan (Varlamov et al. 2002, Haga 2003, Nemtsev et al. 2004, Wu et al. 2004).

In the mushroom industry, a massive amount of the biowaste accumulated during mushroom production and harvesting, mainly consists of stalks and mushrooms of irregular dimensions and shapes. The amount of biowaste obtained can be up to approximately 50,000 metric tons of waste material per year (Wu et al. 2004). These materials have been used as a raw material for the

production of chitin and chitosan because they can be obtained under a controlled environment all year round (Wu et al. 2004). Currently, there is only one company involved in commercial production of chitosan from mushroom, i.e., Belgium Company, Kitozyme (Roberts 2008).

Therefore, this article reviews the recent development of the production process of chitin and chitosan from insects, terrestrial crustaceans, and mushrooms. Moreover, the possibility for the large-scale production of chitin and chitosan from these sources are discussed.

1.2 CHITIN AND CHITOSAN FROM INSECTS AND TERRESTRIAL CRUSTACEANS

1.2.1 Composition and Structure of Organic Matrices of Insects and Terrestrial Crustaceans

The composition and biosynthesis of chitin have been studied in insects such as mosquitoes, cockroaches, honeybees, silkworms, *Drosophila melanogaster*, *Extatosoma tiaratum*, *Sipyloidea sipylus*; in terrestrial crustaceans such as *Armadillidium vulgare*, *Porcellio scaber*; and in nematodes (Anantaraman and Ravindranath 1976, Carlberg 1982, Veronico et al. 2001, Nemtsev et al. 2004, Moussian et al. 2005, Tauber 2005, Paulino et al. 2006, Hild et al. 2008). The composition and structure of their organic matrices are different from each other.

The organic matrices of honeybees are composed of 23%–32% of chitin, 35%–45% proteins, 30%–40% melanin, and 3% mineral compounds (Nemtsev et al. 2004). The organic matrices of silkworms are composed of about 20% of chitin and others components such as proteins, minerals, and fat (Zhang et al. 2000). Chitin is one of the major components of the cuticle, tracheae, and peritrophic matrix (Nemtsev et al. 2004, Arakane et al. 2007 and Kramer and Koga 1986, cited in Kato et al. 2006). In cuticle, some of the chitin is covalently linked to the protein. Several different types of chitin–protein assembly in the insect cuticle have been recognized (Rudall 1965). The chitin fibers were found in nanometer scale (Chen et al. 2004). Two types of chitin, rod and sheet, are present in insect cuticle (Rudall 1967). The cuticle of insects consists of four distinct layers: an outer epicuticle followed by an exocuticle, an endocuticle, and an innermost epidermis layer. The major roles of chitin in insect cuticle are (1) to attach the cuticle to the epidermal cells, thereby maintaining epidermal morphology; (2) to integrate and support the assembly of the epicuticle; (3) to stabilize the layered organization of the cuticle; and (4) for cuticle pigmentation (Moussian et al. 2005). The cuticle of fly larvae practically does not contain melanin; however, the cuticle of adult insects is composed of chitin–melanin and melanoprotein complexes (Nemtsev et al. 2004, Kurchenko et al. 2006). The schematic interpretation of the organic matrix of insect cuticle is shown in Figure 1.1.

Chitin in terrestrial crustaceans is associated with calcium carbonate, proteins, lipids, and pigments (Luquet et al. 1996, Hild et al. 2008). The structure of the cuticle of terrestrial crustaceans

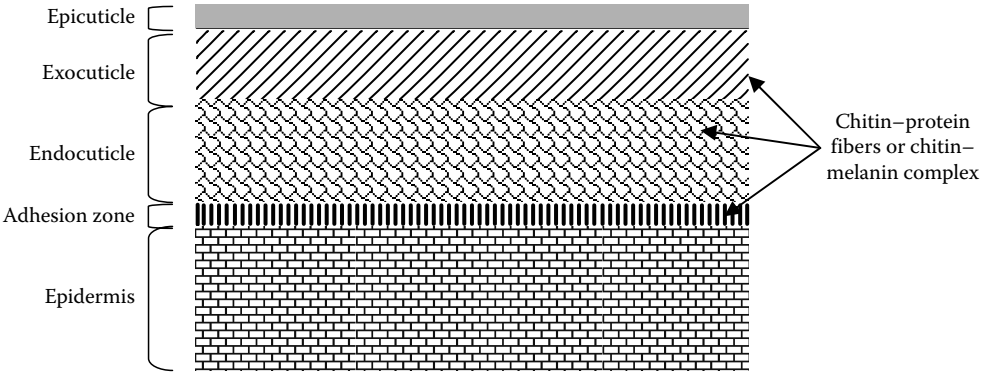

FIGURE 1.1 Schematic interpretation of organic matrix of insect cuticle (not drawn to scale).

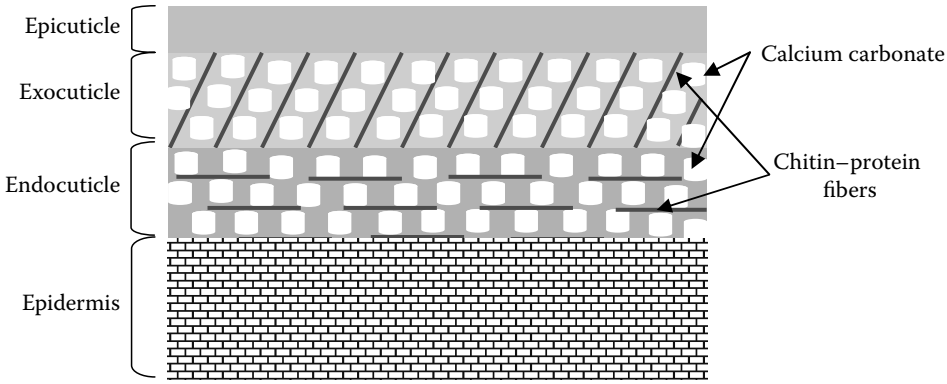

FIGURE 1.2 Schematic interpretation of organic matrix of terrestrial crustacean (not drawn to scale).

is same as insect cuticle, but the composition of the four distinct layers are different. These layers of organic matrix are composed of chitin–protein fibers that are associated with various amounts of calcium carbonate (Hild et al. 2008). The outermost layer, epicuticle, is not mineralized and the exocuticle and endocuticle are constructed with rows of calcium carbonate granules aligned along parallel chitin–protein fibers (Hild et al. 2008). This type of mineralized structure does not contain a great amount of insoluble proteinaceous components (Luquet et al. 1996). The organic matrix of terrestrial crustacean is shown in Figure 1.2.

In *E. tiaratum*, chitin contains 24% in exuviae, 3.4% in whole eggs and 3.8% in eggshells (Carlberg 1982). In *S. sipylus*, 14% of the body consists of chitin (Carlberg 1982). In the exoskeleton of the cockroach, presence of chitin is about 30%–37% in the dosal abdomen, ventral abdomen, metathoracic legs, mesothoracic legs, prothoracic legs, pronotum, head; about 29%–25% in genitalia, dorsal thorax, ventral thorax, antennae, cerci; and about 19% in forewings and hindwings (Tauber 2005). Moreover, chitin also presents in the egg shells of aeanthors (Anantaraman and Ravindranath 1976).

1.2.2 Extraction of Chitin and Chitosan from Insects

Insect cuticle is composed of chitin, melanin, and protein, of which protein and melanin are alkali-soluble (Nemtsev et al. 2004). The procedure for extraction of chitin and chitosan from the cuticle of insects is similar to that of crustacean sources, where the procedure includes demineralization, deproteination, decolorization, and deacetylation (Haga 1996, Zhang et al. 2000, Varlamov et al. 2002, Nemtsev et al. 2004, Paulino et al. 2006). Their demineralization studies were carried out using 1–2 N HCl for 0.3–96 h at 25°C–100°C, which is stronger than the demineralization process of aquatic crustacean materials. The demineralization of shrimp waste is completed within 15 min using 0.25 M HCl at room temperature (Roberts 2008). Zhang et al. (2000) found that the crystallinity of chitin increased and 55% of the *N*-acetyl groups of silkworm chitin were removed after treatment with 2 N HCl at 100°C. Therefore, the treatment of insect cuticle with dilute HCl is not only for removal of mineral but also for removal of the acetyl groups of insect chitin. The deproteination of insect pupa and larva was carried out by using 0.75–2.5 N NaOH for 2–42 h at 40°C–100°C, which is similar to that of crustacean raw materials. In which, Zhang et al. (2000) washed again the chitin sample with 0.4% Na_2CO_3 for 20 h to completely remove the proteins. The deacetylation of insect chitin was carried out by using 10–12.5 M NaOH for 15–16 h at 110°C–150°C.

Paulino et al. (2006) obtained chitin with high purity from silkworm pupa, but the yield of chitin and chitosan were low when compared with the chitin and chitosan produced from aquatic crustacean shells. The lower yield of chitin may be due to the effect of treatment on insect materials with HCl at high temperature (Table 1.1). Therefore, it should be considered that the process of acid demineralization step is to be carried out under mild condition, which can avoid the possibility

TABLE 1.1
Procedure for the Extraction of Chitin and Yield of Chitin from Insects

	Treatment Conditions						
	Deproteination			Demineralization			
Species	NaOH Conc. (M)	Temp. (°C)	Time (h)	HCl Conc. (M)	Temp. (°C)	Time (h)	Yield of Chitin (%)
Silkworm pupa (Paulino et al. 2006)	1	80	24	1	80	24	2.59[a]
Silkworm pupa (Haga 1996)	1	Boil	42	2	25	96	33
Dry dead bees (Varlamov et al. 2002)	2.5	—	—	—	—	—	10–20[b]
Bee corpses (Nemtsev et al. 2004)	1	40	2	—	—	—	25[b]

[a] High-purity chitin.
[b] Chitin associated with melanin.

TABLE 1.2
Procedure for the Extraction of Chitosan and Yield of Chitosan from Insects

	Treatment Conditions						
	Decolorization			Deacetylation			
Sources	NaOH Conc. (M)	Temp. (°C)	Time (h)	NaOH Conc. (M)	Temp. (°C)	Time (h)	Yield of Chitosan (%)
Chitin from silkworm pupa (Haga 1996)	EtOH	—	4	12.5	150	16	32.7
Chitin from bee corpses (Nemtsev et al. 2004)	H_2O_2	75–80	1	12.5	125	1.5	16–25[a]

[a] Chitin associated with melanin.

of acid hydrolysis of insect chitin. Haga (1996) and Nemtsev et al. (2004) have used decolorization agents to remove pigment compounds from chitin of silkworm pupa and bee corpses (Table 1.2). Nemtsev et al. (2004) observed that melanins were absent in the chitin, which is called white chitin, after treatment with hydrogen peroxide. The degree of deacetylation of insect chitosan was about 70%–95% (Nemtsev et al. 2004).

1.3 CHITIN AND CHITOSAN FROM MUSHROOMS

1.3.1 Compositions and Structure of Cell Wall of Mushroom

The basidiomycota, or club fungi, possess separate hyphae and they produce mushrooms (Tortora et al. 1995). There are three main parts in a mushroom: pileus, stipe, and mycelia. In the cell wall of mushroom, microfibrils are arranged in a triple-helical tertiary conformation (Kamada et al. 1991, Synytsya et al.

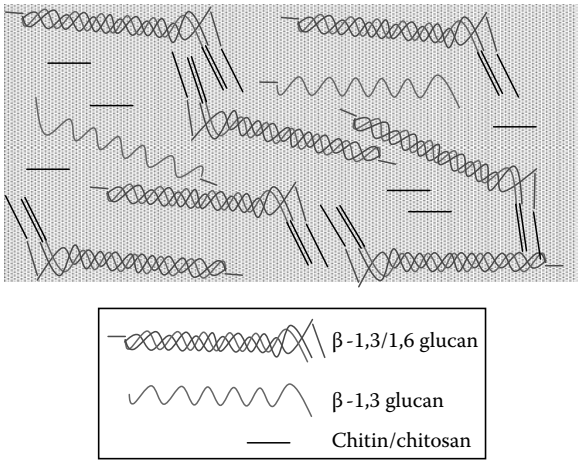

FIGURE 1.3 Structure of cell wall of a typical mushroom (not drawn to scale).

2009). The main components of mushroom are water, proteins, chitin, chitosan, and glucans (Zivanovic et al. 2003, Mario et al. 2008). Dry mushroom contains glucose (80%–90%), mannose (3%–7%), galactose (2%–6%), xylose (0.5%–1%), arabinose (0%–1%), uronic acid (2%–3%), protein (20%–25%), chitin (10%–20%), vitamins, and trace amounts of ash (Tsujiyama 1999, Mario et al. 2008, Synytsya et al. 2009). After removal of protein from mushroom, a high quantity of glucans and a small quantity of ash is present in the mushroom chitin, but proteins were absent (Wu et al. 2004, Yen and Mau 2007a,b, Mario et al. 2008). The highly branched β-1,3/1,6-glucans are positioned at cell surface and β-1,3-glucan linear forms are lined to crystalline chitin, and α-1,3-glucans serve as amorphous matrix throughout the wall (Angeli-Papa and Eyme 1978; Michalenko and others 1976, Wessels and others 1990, cited in Zivanovic et al. 2003). The structure of cell wall of mushroom is shown in Figure 1.3.

1.3.2 Extraction of Chitin and Chitosan from Mushrooms

The extraction of chitin and chitosan from different species of mushrooms (i.e., *Agaricus bisporus*, *Auricularia auriculajudae*, *Lentinula edodes*, *Trametes versicolor*, *Armillaria mellea*, *Pleurotus ostreatus*, *Pleurotus sajor-caju*, and *Pleurotus eryngii*) obtained from mushroom farm or from cultivation in synthetic medium has been illustrated in few of the publications (Pochanavanich and Suntornsuk 2002, Yen and Mau 2007b, Mario et al. 2008). The mushroom, *P. sajor-caju* showed highest yield of biomass and *L. edodes* was the lowest when compared with other mushrooms under submerged fermentation (Table 1.3). The growth rate of mushroom depends on cultivation conditions such as temperature, moisture content, medium composition and type, and mainly species of the mushroom.

The processes and conditions for the extraction of chitin and chitosan from mushroom were nearly same in the methods of Crestini et al. (1996) and Pochanavanich and Suntornsuk (2002), and were different in Mario et al. (2008) and Yen and Mau (2007a). Crestini et al. (1996) and Pochanavanich and Suntornsuk (2002) used 1 M NaOH at 121°C for 0.25 h for deproteination and the chitosan was extracted from the collected alkaline insoluble material using 2% acetic acid at 95°C for 8–14 h. Mario et al. (2008) used 1 M NaOH at 40°C for 15–17 h for deproteination and the chitosan was extracted from the collected alkaline insoluble material using 5% acetic acid at 90°C for 3 h. The total yield of chitin, i.e., 85–196 mg/g of dried mushroom and only a low yield of chitosan, 10–40 mg/g of dried mushroom were obtained from different species of mushrooms by both chitosan extraction procedures (Table 1.3).

However, Yen and Mau (2007a,b) extracted chitin and chitosan using alkaline treatment, followed by decolorization and then deacetylation with concentrated sodium hydroxide solution. In their process, they used mushroom *L. edodes* from a mushroom farm and they did not purify the chitosan

TABLE 1.3
Yield of Chitin and Chitosan from the Mushrooms Grown under Different Cultivation Conditions

Mushroom Species	Method of Cultivation	Optimal Harvesting Time (Days)	Yield of Biomass (g/L)	Yield of Chitin or *Chitosan (mg/g of Biomass)
L. edodes (Crestini et al. 1996)	SMF	12	~3	*40
A. bisporus (SMR 13) (Mario et al. 2008)	SMF	21	3.5	85
A. auricula-judae (SMR 54) (Mario et al. 2008)	SMF	21	6.8	196
L. edodes (SMR 90) (Mario et al. 2008)	SMF	21	3.2	101
T. versicolor (SMR 117) (Mario et al. 2008)	SMF	21	4.2	131
A. mellea (SMR 439) (Mario et al. 2008)	SMF	21	6.4	111
P. ostreatus (SMR 684) (Mario et al. 2008)	SMF	21	4.9	153
P. eryngii (SMR 755) (Mario et al. 2008)	SMF	21	4.6	87
L. edodes no. 1 (Pochanavanich and Suntornsuk 2002)	SMF	9	1.4	*33
P. sajor-caju no. 2 (Pochanavanich and Suntornsuk 2002)	SMF	21	~10	*12
L. edodes (Yen and Mau 2007)	Farm	—	—	*240 (cg)
L. edodes (Yen and Mau 2007a)	Farm	—	—	283 (cg)

Note: SMF, submerged fermentation; Farm, mushroom farm; cg, chitin/chitosan with other compounds.
*, yield of chitosan (mg/g of biomass).

using acid extraction process. The obtained chitin and chitosan were contaminated with glucan or other polysaccharide.

Moreover, Crestini et al. (1996) reported that the yields of chitosan, viz., 120 mg/L of fermentation medium under liquid fermentation conditions, and 6.18 g/kg of fermentation medium under solid-state fermentation conditions are produced from the mushroom, *L. edodes*. Based on this data, it can be considered that the cultivation of mushroom on solid support, which is the natural growing method of mushroom, might be the best cultivation method for the production of chitin and chitosan from mushrooms. The yield of extracted chitin and chitosan depends on mushroom species, harvesting time, and chitin and chitosan extraction processes and conditions (Pochanavanich and Suntornsuk 2002, Yen and Mau 2007a).

By studying the organic matrix of mushroom (Figure 1.3), it can be considered that the major problem in the extraction of chitosan from mushroom source is that chitin/chitosan is complexed or intertwined with glucan or other polysaccharides. Consequently, the extraction of chitin and chitosan from the resultant suspension is difficult and the yield of chitin and chitosan is very low. However, the knowledge in this area is very limited and it is necessary to solve more problems other than the particular problem mentioned above to reach the final goal of chitosan production from mushroom.

Based on the present knowledge on this topic, the mycelium of basidiomycetes can be considered an alternative source for the production of chitin and chitosan that might be useful for some specific practical applications. Mushroom chitosans have a degree of deacetylation of 70%–90% that depends on mushroom species and treatment conditions, and average molecular weight about 1–2 × 10^5 Da (Crestini et al. 1996, Pochanavanich and Suntornsuk 2002, Yen and Mau 2007a, Mario et al. 2008).

1.4 CONCLUSION AND RECOMMENDATION

Several excellent reviews and research papers for the production of chitin and chitosan have appeared in various journals, international symposia, and conferences on chitin and chitosan. Most of the published papers emphasized the production of chitin and chitosan from aquatic crustaceans and from

fungi in Zygomycetes species. Only few published papers reported on the production of chitin and chitosan from insects and mushrooms and on the composition of chitin in terrestrial crustaceans. Among few published papers, the basic production processes of chitin and chitosan from cuticle of insects and cell wall of mushrooms are similar to the process of production of chitin and chitosan from aquatic crustacean source. The yield of purified chitin and chitosan from these sources was lower than that of aquatic crustacean sources. For these sources, the problem in production process like chitin complexed with other compounds such as melanin (in insect) and glucan (in mushroom) needs to be solved. This makes it difficult to extract and purify the chitin and chitosan from insects and mushrooms. Therefore, until now, there has been almost no attempt at commercialized production of chitin and chitosan from biowaste of insects, terrestrial crustaceans, and mushrooms. While significant progress has been made in recent years on the production of chitin and chitosan from terrestrial organisms, most researchers from structural biology also studied in more detail the compositions and structure of organic matrix of terrestrial organisms. Taken together, it is safe to predict that the final goal of the large-scale production of chitin and chitosan from terrestrial organisms will be reached in the near future.

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2 Chitin and Chitosan from Marine Organisms

Wolfram M. Brück, John W. Slater, and Brian F. Carney

CONTENTS

2.1 INTRODUCTION

Isolated from mushrooms in 1811 by Henry Braconnot (1780–1855), chitin was the first polysaccharide identified by man, preceding cellulose by about 30 years (Braconnot 1811, Labrude and Becq 2003). In 1859, Prof. C. Rouget subjected chitin to alkali treatment, which resulted in a substance that could, unlike chitin itself, be dissolved in acids. The term "chitosan" was given to deacetylated chitin by Hoppe-Seiler in 1894 (McKay 1995). While chitin remained an unused natural resource for a long time, interest in this polymer and its derivatives such as chitosan and chitooligosaccharides (COS) has increased in recent years due to their unique biodegradability, biorenewability, biocompatibility, physiological inertness, and hydrophilicity (Vårum et al. 1991, Kumar 2000, Beaney et al. 2005, Kurita 2006). Worldwide production of chitin has been estimated to be approximately 10^{11} tons per annum making it one of the most abundant natural compounds on earth (Kurita 2006). While chitin occurs in fungi, diatoms, nematodes, arthropods, and a great number of other animals and plants, commercial exploitation has focused on a limited number of species for chitin extraction (Hayes et al. 2008). Shrimps, crabs, lobsters, krill, and squid wastes from the marine processing industry have become the major resource used today (Teng et al. 2001).

Approximately 70% of marine capture fisheries are utilized for processing and a considerable quantity of the processed catch remains as wastes (Kim and Mendis 2006). Discards exceed 20 million tons per annum, which is the equivalent of 25% of the total annual production (FAOSTAT 2001). In crustaceans, waste makes up approximately 45% by weight of the shellfish and comprises discarded heads, thorax, claws, and shells (Subangsinghe 1994, Zakaria et al. 1998). These chitinous shellfish wastes are considered hazardous due to their high perishability and high polluting effect if disposed off-shore (Healy et al. 1994). In the sea, chitinous wastes rapidly lead to eutrophication and exert a high biochemical oxygen demand (BOD), while on land, the waste quickly becomes colonized by pathogens and spoilage organisms causing environmental and public health concerns (Islam et al. 2004, Beaney et al. 2005). The European Union, United States, Japan, and other countries have responded to these issues by setting specific maximum limits for biodegradable municipal wastes that may be disposed in landfills and at sea (Knorr 1984, Shahidi and

TABLE 2.1
Contents of Chitin and Calcium Carbonate in Industrially Important Marine Species

Type	Chitin (%)	$CaCO_3$ (%)	Location
Phylum Crustacea			
Euphausia sp. and *Meganyctiphanes* sp. (Krill)	20–30	20–25	Cuticle/exoskeleton
Chionoecetes sp., *Cancer* sp., and *Carcinus* sp. (Crab)	15–30	40–50	Cuticle/exoskeleton
Paralithodes sp. (King crab)	~35	40–50	Cuticle/exoskeleton
Callinectes sp. (Blue crab)	~14	40–50	Cuticle/exoskeleton
Crangon sp. and *Pandalus* sp. (Shrimp)	17–40	20–30	Cuticle/exoskeleton
Penaeus sp. (Prawn)	~40	20–30	Cuticle/exoskeleton
Nephrops sp. and *Homarus* sp. (Lobster)	60–75	20–30	Cuticle/exoskeleton
Lepas sp. (Goose Barnacle)	~59	20–30	Shell
Phylum Mollusca			
Mytilus sp. and *Pecten* sp., etc. (Mussels, clams, etc.)	~3	85–90	Shell
Crassostrea sp. (Oyster)	~6	85–90	Shell
Loliginidae sp. and *Ommastrephidae* sp. (Squid)	20–40	Negligible	Pen

Sources: Adapted from Tharanathan, R.N. and Kittur, F.S., *Crit. Rev. Food Sci. Nutr*., 43, 61, 2003; Kurita, K., *Mar. Biotechnol*., 8, 203, 2006.

Synowiecki 1991, Healy et al. 2003). This has led to the exploitation of fisheries wastes in high-volume, low-value products such as fishmeal and fish silage, pet foods, and fertilizers (Choudhury and Gogoi 1995, Choudhury and Bubliz 1996). Since crustacean and mollusk waste streams constitute a rich source of (higher) value-added products such as chitin, protein, pigments, and flavor compounds, the full exploitation/bioconversion of this easily accessible resource has attracted much interest. However, the high costs of purification and the production of corrosive wastes associated with traditional industrial methods of extraction have proved problematic (Muzzarelli 1990, Wang and Hwang 2001, Hayes et al. 2008). Proportions of chitin from these sources may vary with season and species but in general, exoskeletons contain 15%–40% chitin, 20%–40% protein, and 20%–50% calcium carbonate, with other components such as pigments, lipids, and other metal salts present as minor components (No and Meyers 1995, Singer and Wooten 2003, Kurita 2006). A list of commercially exploited marine species and their chitin content is shown in Table 2.1. This overview discusses the fundamental aspects of chitin, chitosan, and chitin/chitosan oligosaccharide extraction from these marine sources through chemical, enzymatic, and emerging biotechnological isolation procedures.

2.2 CHITIN, CHITOSAN, AND THEIR OLIGOMERS

Chitin is a cationic amino polysaccharide composed of *N*-acetyl-D-glucosamine (GlcNAc, 2-acetamido-2-deoxy-D-glucose; ~50%–100%) with β (1 → 4) glycosidic bonds between each monomer (Figure 2.1a; Beaney et al. 2005). Chitin occurs in three polymorphic solid state forms designated as α, β, and γ chitin which differ in their degree of hydration, size of unit cell, and number of chitin

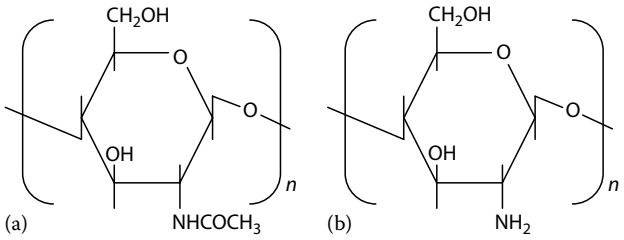

FIGURE 2.1 Molecular structures of (a) chitin and (b) chitosan.

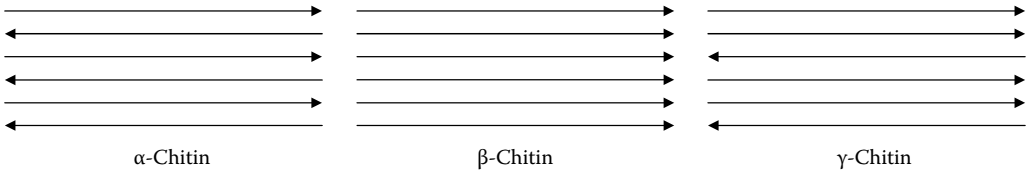

FIGURE 2.2 The structural arrangements of chitin.

chains per unit cell (Einbu 2007). Chains of GlcNAc may be arranged in a tightly compacted crystalline structure of antiparallel sheets and extensive intermolecular hydrogen bonding (α-chitin), in a more mobile allomorph of parallel sheets (β-chitin), or a combination of both (γ-chitin) (Figure 2.2; Carlstrom 1957, Gooday 1990, Kurita 2001, Rudrapatnam et al. 2002). α-Chitin is most abundant and is found in shellfish exoskeletons and fungal cell walls. β-Chitin is mainly found in squid pens and diatoms while γ-chitin may be predominantly found in squid and cuttlefish stomach lining. Isolation methods for the various types of chitin differ widely with α-oligomers requiring harsher methods than β-chitin (Minke and Blackwell 1978). Due to its weaker intermolecular forces, β-chitin is soluble in a variety of solvents and readily swells in water (Blackwell 1969, Abdou et al. 2008). In addition, β-chitin may be readily and irreversibly transformed into α-chitin by steam (Kurita 2001).

When chitin reaches a nitrogen content of more than 7% by weight, or when the degree of deacetylation (DD) is over 60% the term chitosan is preferred (Peter et al. 1986, Gagné 1993). Chitosan is a biopolymer that exists of D-glucosamine (GlcN, ~80%) and GlcNAc (~20%) units obtained through deacetylation of chitin using hot alkali (Figure 2.1b; Tharanathan and Kittur 2003). Like chitin, chitosan is a β (1 → 4) glycan and has been described as "nature's most versatile biomaterial" (Sandford 1989). Whereas other naturally occurring polysaccharides are acidic in nature, both chitin and chitosan are basic and as such undergo the typical neutralization reactions of alkaline compounds (Kumar 2000, Rudrapatnam et al. 2002). Although at a molecular level, chitin and chitosan appear similar, both possessing reactive hydroxyl and amino groups, chitosan is more accessible to reagents which may be due to its less crystalline structure. While there are few solvents for chitin, nearly all aqueous acids readily dissolve chitosan, formic acid and acetic acid being most commonly used (Muzzarelli 1985, Tsugita 1990, Rudrapatnam et al. 2002). Acid hydrolysis at room temperature or 0°C effectively cleaves the glycosidic linkages of the chitin and chitosan main chains, lowering its molecular weight (Kurita 2006). Resulting low molecular weight chitin/chitosan oligomers (COS) are β (1 → 4) linked homo- or heterooligomers of GlcNAc and/or GlcN with a chain length of up to $n = 15$ and a molecular weight of up to 10 kDa (Domard and Cartier 1989, Jeon and Kim 2000, Kim and Rajapakse 2005). Unlike chitin or chitosan, COS have a lower viscosity with greater solubility in water at neutral pH due to their shorter chain length and free amino groups in GlcN units (Kim and Rajapakse 2005). These aspects have made them attractive targets for applications the medical, food, and biotechnological fields (Xie et al. 2002, Kim and Rajapakse 2005, Einbu 2007).

The production of chitin and derivatives from shellfish waste is largely dependent on the chitin content of utilized shell which varies with different species due to environmental factors, stage of maturation, feeding, and harvesting conditions. Evidence further suggests that chitin in shellfish forms a microfibril matrix of covalent bonds with α-amino acids such as tyrosine as well as with peptides and cuticular proteins that is hardened by mineral salts (Kozloff 1990, Beaney et al. 2005). Moreover, chitin may form stable glycoproteins by covalently linking to arthropodins, reslins, and sclerotins through aspartyl and histidyl residues (Rudrapatnam et al. 2002). As a result, harsh conditions for the extraction of chitin from marine species are generally required.

2.3 METHODS OF EXTRACTION FROM MARINE SOURCES

The isolation of chitin from shellfish waste consists of three steps: deproteinization (DP), demineralization (DM), and decolorization (DC) whereby the order of the first two steps is generally considered irrelevant if protein or pigment recovery is not an objective (Shahidi and Synowiecki 1991). Chitin is further deacetylated (DA) to make chitosan or other products for a wide array of applications. Both chemical and enzymatic non-continuous batch methods are widely used on an industrial scale for the production of chitin, chitosan, and COS.

2.3.1 Chemical Extraction

Several procedures for the preparation of chitin and chitosan from different shellfish wastes have been developed over the years, some of which form the basis of the chemical processes used for the industrial production of chitin and derivatives (Fernandez-Kim 2004). A representation of current industrial chitin processes are summarized in Figure 2.3. Industrial techniques for chitin and chitosan extraction from different shell waste streams normally rely on harsh chemical processes due to covalent associations with other shell constituents. These methods generate large quantities of hazardous chemical wastes and partial DA of chitin and hydrolysis of the polymer may occur, leading to inconsistent physiological properties in the end products (Andrade et al. 2003, Kim and Mendis 2006).

A detailed review of the different chemical extraction methods for chitin from shellfish waste available between 1954 and 1993 was performed by No and Meyers (1995). Similarly, various methods for DM and DP of chitin from Hariana Shrimp (*Metapenaeus monoceros*) shells were examined by Naznin (2005). In general, proteins are first removed from ground shells by treating with mild sodium hydroxide or potassium hydroxide solution at elevated temperature. Alkali concentrations usually between 1% and 10% with temperatures ranging from 30°C to 100°C, independent of starting material, are most common. Typical reaction times used vary between 30 min and 12 h. For example, Whistler and BeMiller (1962) extracted protein using a 10% NaOH solution at 100°C for 72 h to effect depolymerization and deacetylation. The optimal method for the DP of crab shell and shrimp shell waste was reported to be 1%–2% KOH at 90°C with a shell to alkali solution ratio of 1:20 (w/v). A minimum period of 1 h was needed to extract 90% of the proteins, with 2 h removing most proteins in the shells (Shahidi and Synowiecki 1991).

The removal of calcium carbonate, calcium phosphate, and other mineral salts found in shell waste is accomplished by extraction with dilute acids. It has been shown to be important that the amount of acid is stoichiometrically equal to or greater than all the minerals present in the shell to ensure complete DM (Sahidi and Synowiecki 1991). Hydrochloric acid at room temperature and a reaction time of 2–3 h was most commonly used even though DA and chain hydrolysis may be observed (No and Meyers 1995). In order to minimize modifications of the native chitins, the use of ethylenediaminetetra acetic acid (EDTA) for DM or combined DP and DM has been suggested (Foster and Hackman 1957, Austin et al. 1981).

Commonly used industrial-scale DP and DM of shellfish wastes produce a brown to brownish-white product that may be bleached using a variety of reagents while removing residual lipids.

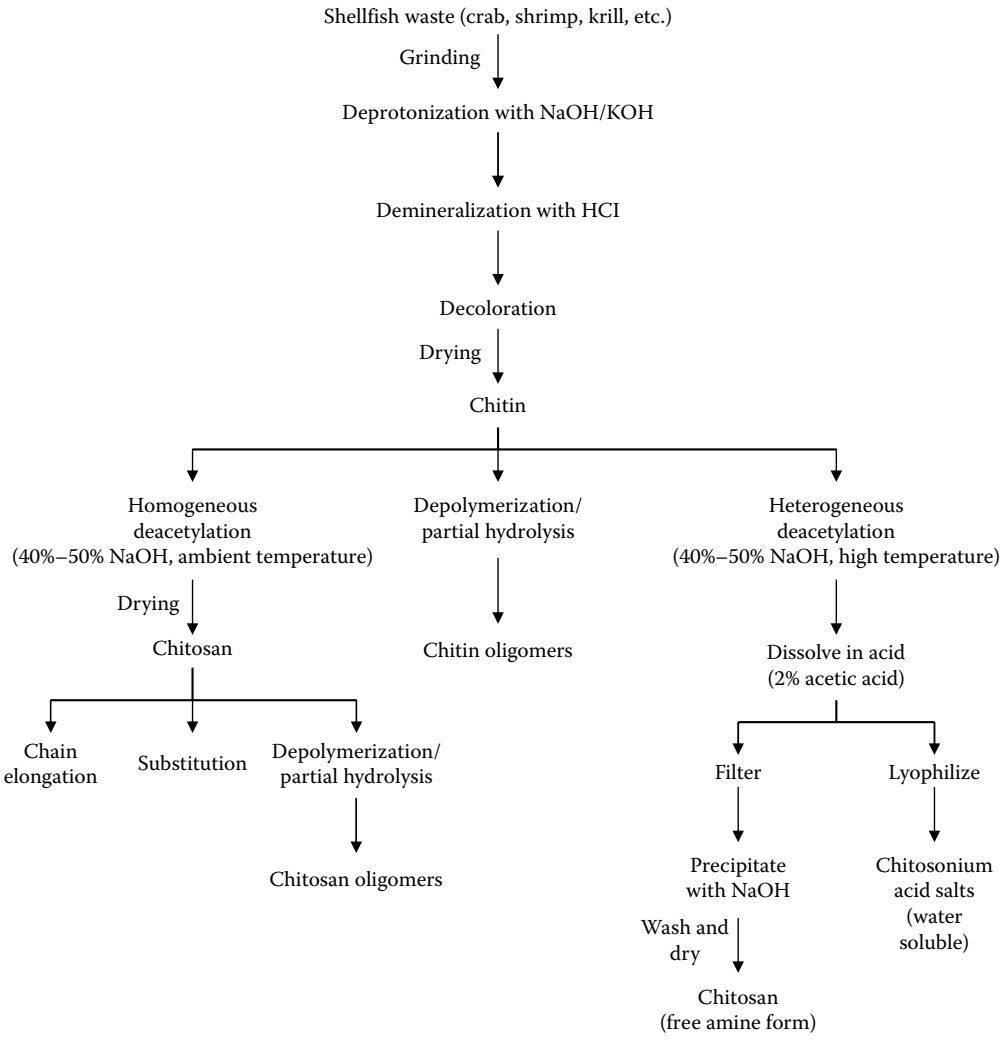

FIGURE 2.3 Chemical manufacturing processes for chitin, chitosan, and COS.

Most commonly used reagents include ethanol, ether, acetone, sodium hypochlorite, hydrogen peroxide, or a combination, chiefly used at ambient temperature (No and Meyers 1995). White chitin and chitosan without the need for DC was obtained by using 30% HCl for DM and 1.5 N NaOH for DP from Hariana Shrimp (Naznin 2005).

While chitosan may be produced by DA using highly concentrated sodium-, or potassium hydroxide solutions (40%–50%) at high temperature (100°C of higher) in an inert atmosphere, though a highly deacetylated chitosan is usually not obtained by these methods (Peniston and Johnson 1980, Johnson and Peniston 1982). Higher temperatures reduced the molecular weight of the resultant chitosans (Lusena and Rose 1953). Using several DA methods, it was observed that decreasing the concentration of alkali resulted in an increase in the time required to obtain a soluble, less viscous product, while saturating chitin with alkali had little effect on DA and viscosity. Effective DA of chitin was achieved by washing the intermediate product in two or more changes of water during DP. This resulted in an undegraded, nearly 100% deacetylated, chitosan (Mima et al. 1983). Another method that requires considerably less severe conditions and resulted in better DA with less degradation was described by Domard and Rinaudo (1983) who added thiophenol as an oxygen trap and catalyst during DA. Here, DA took place using a calculated four times excess of NaOH for the

total *N*-deacetylation of all amino groups in chitin (concentration range 2%, w/v). Reaction time was 1 h at a temperature of 100°C. The technique was later refined by Suryanarayano Rao et al. (1987) who impregnated chitin with a four times excess NaOH (w/v) by mixing and heating to 60°C for 2 h. The methods for chitosan production from squid pen are generally much milder due to the relatively weaker intermolecular forces and crystalline state of β-chitin (No and Meyers 1995, Lamarque et al. 2004, Kurita 2006). This was clearly demonstrated by Kurita et al. (1993) who obtained 70% DA from squid pen β-chitin compared to only 20% using shrimp shell α-chitin using 30% NaOH at 100°C for 2 h. Conversely, chitosan with a DD of 90% was obtained from squid pens after autoclaving for 15 min (Abdou et al. 2008).

Rout (2001) evaluated the effects of reversing the first two steps (DP and DM) or reducing the number of steps on the fat and water binding capacities of chitin and chitosan from crab and crawfish exoskeletons and found a high fat-binding capacity when the processing steps were reduced. Similarly, No et al. (2002) eliminated the DP step and reduced the treatment time with alkali to examine their effects on the physiochemical and functional properties of chitosan. Results showed that the end product had a higher molecular weight and viscosity, a lower DD, lower solubility and reduced water and fat-binding capacity when compared to chitosan obtained by traditional methods. A more comprehensive study on the influence of chemical processes on the production of chitosan from crawfish wastes was performed by Fernandez-Kim (2004). These results indicated that modification in the order of the processing steps yielded chitosans with different characteristics. The most noteworthy change was observed with crawfish chitosan that had a processing order of DM, DP, DA, DC and where properties of a light polyelectrolyte were observed. In processes that started with DA, poor yields were observed.

For the large-scale chemical production of COS, crab and shrimp shell wastes are currently used as the major source (Kim and Rajapakse 2005). Chitin and chitosan may be hydrolyzed with concentrated HCl at elevated temperatures to form monomeric glucosamine, while less severe methods yield a series of GlcNAc oligomers (chitin) or GlcN oligomers (chitosan) (Domard and Cartier 1989, Kurita 2006). However, the chemical hydrolysis of chitin or chitosan often results in low yields of COS with desired molecular weights and a large amount of monomers (Uchida et al. 1989). Additionally, toxic compounds may also be produced during the reaction, increasing the risk of environmental pollution and making a chemical production unattractive for the production of bioactive COS for human use. Therefore, enzymatic processes for COS production are now preferred (Kim and Rajapakse 2005).

2.3.2 Enzymatic/Biotechnological Methods

An interesting alternative to chemical methods is the use of biotechnological processes for chitin, chitosan and COS preparation (Figure 2.4), however, residual protein often remains high and reaction times are significantly increased compared to chemical methods. Enzyme costs are furthermore prohibitive, limiting enzymatic methods in industrial applications unless the process is made more efficient (Percot et al. 2003). Commercially available proteolytic enzymes such as Alcalase (EC 3.4.21.62), chymotrypsin (EC 3.4.21.2), and papain (EC 4.3.22.2) have been used to extract protein and chitin from shell waste (Wang and Chio 1998, He et al. 2006). Wang et al. (2008) used stem bromelain (EC 3.4.22.32) and papain to hydrolyze chitinous materials from squid pen to produce COS. Bromelain showed far better hydrolysis than papain, with an optimum concentration of 0.1%. Alcalase digestion from shrimp (*Crangon crangon*) waste allowed the recovery of chitin with approximately 4.4%–7.9% residual protein content (Synowiecki and Al-Khateeb 2000). Duarte de Holanda and Netto (2006) recovered chitin, protein and astaxanthin from the shell waste of the commercial shrimp species *Xiphopenaeus kroyeri* using Alcalase or pancreatin with an enzyme/substrate ratio of 3% and a temperature of 60°C. DP with Alcalase was shown to be more efficient than pancreatin though residual protein content was about twice as high compared to commercial product treated with NaOH.

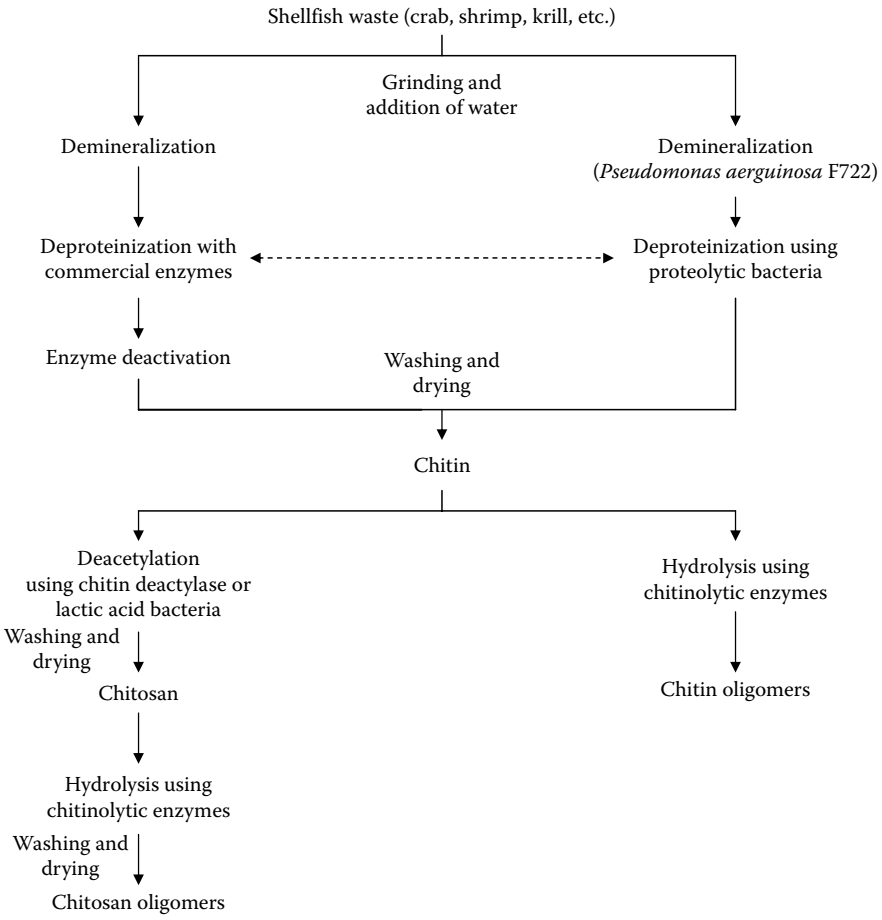

FIGURE 2.4 Biotechnological processes for chitin, chitosan, and COS production.

The use of proteases from bacterial isolates was reported by Shimahara and Takiguchi (1988) as well as Tsai et al. (2002) who used a bacterial protease from *Pseudomonas maltophilia* for DP without alkali. After 24 h, the remaining protein content of crustacean shell was about 1%. Similarly, a protease from *Bacillus* sp. TKU004 was employed at optimized conditions in a 2% slurry of squid pen powder (Wang et al. 2006). DP was 63% after the second day and peaked at 73% after the third day.

Fermentations with bacteria producing proteolytic and chitinolytic enzymes has been researched as an alternative with varying levels of success (Zakaria et al. 1998, Bautista et al. 2001, Jung et al. 2005). Oh et al. (2007) used an organic acid and protease producing soil isolate of *Pseudomonas aeruginosa* F722 to DM and DP crab shell waste samples. At an optimal fermentation temperature of 30°C and with 10% glucose supplementation and 7 days incubation, DM was 92% and DP was 63% after 7 days of incubation. A positive relationship between DM and glucose supplementation was observed. Lactic acid fermentation for the extraction of chitin has been reported for shrimp waste (Shirai et al. 2001, Pacheco et al. 2009), crayfish exoskeleton (Bautista et al. 2001), and prawn waste (Shirai et al. 1998, Zakaria et al. 1998). Prawn (*Nephrops norvegicus*) waste fermented by *Lactobacillus paracasei* or a combination of *L. acidophils, Enterococcus faecium*, and *Pediococcus acidilactici*, yielded a low quality chitin (Zakaria et al. 1998, Beaney et al. 2005). However, DA using lactic acid fermentation yielded chitosan with similar physiochemical properties to those of chemically extracted chitosans (Beaney et al. 2005). No significant difference was observed in chitin yields from shrimp (*Penaeus semisulcatus*) using cultures of *L. plantarum*,

L. acidophilus, and *L. rhamnosus* supplemented with 1% $Fe(NO)_3$ as a mineral nitrogen source (Khanafari et al. 2008). An important effect of operational temperatures on the DM and DP was observed with 30°C–40°C considered most favorable for fermentation of shrimp waste using *L. plantarum* (Pacheco et al. 2009).

Chitin deacetylase from *Colletotrichum lindemuthianum*, *Mucor rouxii*, *Abisidia butleri*, or *Aspergillus nidulans* may convert chitin from shell waste to chitosan (Kauss and Bauch 1988, Arcidiacono et al. 1989, Tsai et al. 2002). However, different preparation methods result in different DD, distribution of acetyl groups, chain length, and conformational structures (Rudrapatnam et al. 2002, Kurita 2006, Synowiecki 2007).

Chitinolytic enzymes such as chitinase, chitonsanase, papin, and lysozyme are widely distributed in all kingdoms and enzymatic hydrolysis for COS production is preferable since these methods obtain greater yields of oligomers with a higher degree of polymerization (Uchida et al. 1989, Muzzarelli et al. 1994, Jeon and Kim 2000, Chen et al. 2003, Kim and Mendis 2006). Endochitinases (EC 3.2.1.14) and chitosanase (EC 3.2.1.132) hydrolyze chitin by random cleavage, generating oligosaccharides such as chitotetraose, chitotriose, and diacetylchitobiose (Hayes et al. 2008). Chitosanase from *Bacillus* sp. has previously been used to hydrolyze deacetylated chitosan in batch fermenters (Izume and Ohtakara 1987, Jeon and Kim 2000). Similarly, *Streptomyces kurssanocii* has been reported to ferment chitosan from Kamchatka-peninsula-shelf-crab (*Paralithodes camtschaticus*) shell. Here, repeated hydrolysis at pH 4.6 and pH 6.2 first lead to a 22–24 kDa chitosan and then to 2–9 kDa acid-free water soluble COS (Ilyina et al. 2000). Even though microbial chitosanases are excellent for COS production, they are considered too expensive for industrial scale production (Kim and Rajapakse 2005). Thus, other sources are generally considered to produce COS at low cost (Zhang et al. 2006).

2.3.3 Emerging Technologies

While most processes for the production of chitin (and derivatives) from shellfish wastes entail a batch production using chemical, enzymatic, or fermentative methods, some research on novel techniques has been performed.

Mahlous et al. (2007) investigated the influence of gamma irradiation on the extraction of chitin and chitosan from prawn shells. Heads and shells from Algerian coast prawn (*Aristens antennatus*) were collected, dried at 60°C and cut into small pieces which were then irradiated at a dose of 75 Gy/min to a dose of 25 kGy. Irradiation reduced the time needed for deproteinization from 3 to 1 h using 1 N NaOH and a reaction temperature of 85°C. Other factors or the DD for chitosan were not influenced.

Concurrent production of chitin from shrimp shells and fungi (three *Aspergillus niger* strains) aimed to utilize the release of fungal proteases to facilitate the deproteinization of washed and demineralized shrimp shell powder and the release of hydrolyzed proteins (Teng et al. 2001). The hydrolyzed protein in turn was used as a nitrogen source for fungal growth, which led to a reduction in pH of the fermentation medium, enhancing the demineralization of the shrimp shell powder. The researchers concluded that fungal fermentation might be a better alternative than costly, commercial protease enzymatic methods that only deproteinize but not demineralize shrimp shells.

A method for the extraction of partially purified chitin from exoskeletons of marine crustaceans by physical means was patented by Singer and Wooten (2003). First, wet shells were cut into particles less than ¼ inch (6.35 mm) which allowed the removal of inherent proteins, minerals, and fat by washing with water and agitated sieving through an 800 µm mesh. The residual chitinous shell particles were then mixed with water to form a concentrated slurry which was passed through a high impact cutting device to yield particles with an average size of approximately 70 µm. Filtering the slurry through a 200 µm mesh removed remaining fat and protein residues with a majority of mineral granules, leaving an extract of approximately 65% chitin.

While the vast majority of chitin/chitosan/COS preparations rely on batch processing methods, a process for the continuous chemical production of chitin from shellfish wastes has been patented (Blum et al. 2000). Using a series of three rotary auger-containing columns allows the serial DM, DP, and washing of shells to produce chitin. Shells are introduced into the first column containing 20% or 25% HCl at 15°C with 1 h dwell time before the remaining acid is washed out with water. The demineralized solids are then moved to the second column containing hot NaOH (80°C). DP time occurs for a 1–2 h duration before solids are moved on to the third column for washing. A fourth column containing hot NaOH (90°C) may be added if the objective is the production of chitosan.

For the continuous enzymatic production of COS, an ultrafiltration reactor has previously been employed (Jeon and Kim 1999). While continuous processes have several advantages such as higher efficiencies and greater enzyme productivity, it was found that the high viscosity of chitosan restricted a continuous operation due to membrane fouling (Allan and Hadwiger 1979, Hadwiger and Beckman 1980, Jeon and Kim 1999). Consequently, a dual reactor system consisting of an ultrafiltration membrane reactor and a column reactor packed with immobilized chitosanase from *Bacillus pumilus* was designed by Jeon and Kim (2000). Here, partially hydrolyzed chitosan was produced from viscose chitin in the column reactor and was fed into the ultrafiltration reactor using an optimal outflow rate of 5 mL/min. No variation of transmembrane pressure was observed in the system indicating that COS production was possible without membrane fouling. In addition, hydrolyzed oligomers could readily be fractionated by passage through different ultrafiltration membranes making it possible to control the molecular weight distribution of COS fractions. While these continuous processes were developed using commercial grade chitosan of unknown origin, it is envisaged that these methods may be applied directly to shellfish chitin and chitosan in the future.

2.4 CONCLUSIONS

The efficient utilization of crustacean and mollusk shell waste streams for conversion to added value products has lead to significant R&D efforts to investigate novel uses for chitinous compounds through new modification reactions. In recent years, this had yielded not only a variety of highly purified and diverse products based on chitin, chitosan and COS, but also delivered a promising solution to the disposal of shellfish wastes. However, the process may still be significantly improved through advances in microbial fermentation and enzymatic hydrolysis technologies as well as improvements in downstream processing. The use of reagent recycling in continuous production methods may also minimize the impact of hazardous chemical effluents ensuing from the chemical derivatization of shell waste. While a preparation of chitin, chitosan, and COS purely by enzymatic means may not be feasible in large-scale industrial production, a combination of chemical and enzymatic reactions may provide a cost effective and environmentally friendly compromise.

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3 Chitin and Chitosan from Microorganisms

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CONTENTS

3.1 INTRODUCTION

Today, a major concern about the use of crustacean chitin/chitosan in the pharmaceutical and biomedical field is still the molecular variability of a natural product, which results in a wide unpredictable range of physicochemical properties. Concurrently, the production of chitin/chitosan from microbial sources appears promising because the process can be manipulated to obtain a pure, rather uniform product with specific characteristics. In addition, the fermentative production of fungi on cheap industrial by-products and wastes is an unlimited and, in principle, a very economic source of chitin/chitosan. As well, the feasibility of obtaining β-glucan from the myceliar chitosan-glucan complex, and the simultaneous extraction of chitin and chitosan make the microbial process more interesting (Pomeroy et al. 2001). This chapter highlights the research on microbial chitin and chitosan with a special focus on major sources and methods of their biotechnological production, as well as the difficulties in utilizing and processing them for selected applications.

3.2 OCCURRENCE OF CHITIN AND CHITOSAN IN THE MICROBIAL WORLD

Chitin is more widely distributed in the microbial world than chitosan and can be found in fungi, molds, yeasts, some ciliates, and chrysophyte algae, even in a few bacteria including streptomycete spores and the stalks of some prosthecate bacteria (Gooday 1990). Fungal cell walls and septa of *Ascomycetes*, *Zygomycetes*, *Basidiomycetes*, and *Deuteromycetes* contain mainly chitin (Peter 2002). It is probably a universal component of fungal walls serving as a fibrous strengthening element responsible for cell wall rigidity. Fungi that were considered to lack chitin, such as many *Oomycetes* and the fission yeasts, have been shown to have small amounts (Dietrich 1973). However, the fungal cell wall contains variable amounts of chitin. In many systems chitin is a major constituent of the cell wall, while in others, it is involved only in cell division or reproductive

structures and is virtually absent otherwise. A family of integral membrane proteins, called chitin synthases, are responsible for the synthesis of linear chains of chitin (β-1,4 *N*-acetylglucosamine) from the substrate UDP-*N*-acetylglucosamine (Munro and Gow 2001).

Chitin can occur in several polymorphic forms, but α-chitin is the most common form found in fungal walls. In α-chitin, the individual chains are aggregated into microfibrils, with hydrogen bonds holding adjacent chains arranged in an anti-parallel manner, making a very strong, rigid structure. Chitin does not occur by itself and it is often accompanied by other polysaccharides, notably glucan, which provides its special properties useful for many applications (Surarit et al. 1988).

The chitin and chitosan contents of fungal cell walls varied between different species of fungi from 2% to 60% of dry mycelia. The content of chitinous compounds could vary even between species of the same genus as illustrated in *Cunninghamella* genus where the chitosan content ranged from 2.3% to 12.9% (Amorim et al. 2003). It seems that the cell wall's chitin content is generally higher in Zygomycetes, particularly in *Mucor rouxii*, *M. mucedo*, *M. circinelloides* (*M. javanicus* IFO 4570), *Rhizomucor miehei*, *Rhizopus oryzae*, *Phycomyces blakesleeanus*, and *Cunninghamella elegans* (Andrade et al. 2000, 2003, Chung et al. 1994, Stamford et al. 2007). Other promising sources of chitin are Ascomycetes and Basidiomycetes containing significant quantities of chitin and acidic polysaccharides as cell wall components (26%–65%) (Synowiecki and Al-Khateeb 2003). However, the mycelia, and the caps and stalks of fruiting bodies of four edible mushrooms, *Lentinus edodes*, *Lycophyllum shimeji*, *Pleurotus sajor-caju*, and *Volvariella volvacea*, contain chitin as a minor component (Cheung 1996).

By contrast, chitosan occurs as a major component in walls of Zygomycetes, but probably is a minor component in many other fungi. It appears to be principal fiber polymer of the cell wall in *M. rouxii* in addition to chitin (Synowiecki and Al-Khateeb 1997). However, depending on culture conditions, in this fungi, chitosan yield reported by different authors widely varied from 8.9% to 35% (Arcidiacano et al. 1989, Knorr et al. 1989, White et al. 1979). The search for more productive sources of chitin has brought to attention the other Mucoralean strains including *Syncephalastrum racemosum* or *Cunninghamella echinulata*, whose call walls contain a rather high amount of chitosan (Amorim et al. 2006). Screening different Zygomycetes strains, Tan et al. (1996) observed that *C. echinulata* exhibited the highest chitosan yield of 7% per mycelia dry weight under optimal conditions. There are only a few reports on the presence of chitosan in Basidiomycetes in *Lentinula edodes* (Crestini et al. 1996) and *Pleurotus sajor-caju* (Pochanavanich and Suntornsuk 2002).

3.3 BIOTECHNOLOGICAL PRODUCTION

The cultivation of selected fungi has attracted attention as a potential method for the chitin/chitosan production because the fermentation process can continue throughout the year and can be manipulated to obtain a product with specific characteristics. Fungal mycelia wastes from biotechnological plants accumulated in the mushroom production and fermentation industries such as waste mycelia of *Aspergillus niger* from a citric acid production plant deserve particular attention as alternative sources of chitin/chitosan materials (Cai et al. 2006, Muzzarelli et al. 2004). However, they are not produced commercially at the large scale due to the low yields obtained until now compared to the other fermentation processes and the variability in the polymer physicochemical properties.

In order to improve microbial production, serious attention was given to determine the effect of fermentation conditions on the production yield and on the polymers physicochemical properties. Generally, the yield and composition of the polysaccharide depend on the microbial species used, age of the producing microbial cells and growth, cultivation medium, and conditions. The chitinous compounds content also depends on the type of fermentation and extraction method. An increase in chitinous material can be obtained either by increased biomass yield or by an increase in the cell wall content of chitin/chitosan. A summary of the advantages and disadvantages of several biotechnological possibilities to produce chitin/chitosan can be found in Table 3.1.

TABLE 3.1
A Summary of the Major Microbial Sources, Cultivation Conditions, and Type of Fermentation of Several Biotechnological Possibilities to Produce Chitin/Chitosan

Microbial Source	Cultivation Media	Cultivation Conditions, Type of Fermentation	Yield, % Based on Dry Mycelia Weight	Molecular Weight/DA	Description	Reference
M. rouxii	YPG medium	SmF, Erlenmeyer flasks, 28°C, 400 rpm	4%–8% chitosan	—/6%–0% for chitosan	Among tested acids, HCl extraction results in higher yields of chitosan with little or no acetyl content and lower DP	White et al. (1979)
M. rouxii	YPG medium	SmF, Erlenmeyer flasks, 28°C, 170 rpm, 48 h	16.4% AIF 8.9% chitin 7.3% chitosan	—/27.3% for chitosan	The method for chitin/chitosan isolation from fungal mycelium was developed	Synowiecki and Al-Khateeb (1997)
M. racemosus *C. elegans*	YPG medium	SmF, Erlenmeyer flasks, 28°C, 100 rpm, 96 h	3.5% chitosan 2.0% chitosan	—/49% for chitosan —/20% for chitosan	In both fungi, the highest yield of chitosan was found in 24 h of cultivation	Amorim et al. (2001)
Aspergillus niger *M. rouxii*	YPG medium	SSF, Erlenmeyer flasks without shaking, 28°C, 9 or 21 days	24.01% chitin 13.25% chitin, 12.49% chitosan	—/76.53% for chitin —/50.07% for chitin —/19.5% for chitosan	The crystallinity of fungal chitin and chitosan was estimated to be less than those from shrimp shells	Wu et al. (2005)
M. circinelloides (*M. javanicus*)	Medium based on D-glucose, L-asparagine and thiamine	SmF, Erlenmeyer flasks, 25°C, 120 rpm, 72 h	23.9% chitin	N/A	The highest chitin yield was obtained with media containing 60 g/L glucose, 3 g/L of asparagine, and 0.008 mg/L thiamine	Andrade et al. (2003)
M. rouxii	MSM medium PDB medium YPG medium	SmF, Erlenmeyer flasks, 30°C, 120 rpm	6.0%–7.7% chitosan	24.8 kDa/12.8% 45.8 kDa/10.2% 55.9 kDa/17.2%	The yield and quality of chitosan from MSM is higher than that from PDB or YPG	Chatterjee et al. (2005)
C. blakesleeana *R. delemar* *A. coerulea* *M. rouxii* *M isabelina*	YM medium	SmF, Erlenmeyer flasks, 27°C, 48 h, 3000 rpm	9.4% chitosan 7.1% chitosan 10.4% chitosan 1.2% chitosan 6.7% chitosan	140 kDa/35% 120 kDa/32% 450 kDa/5% 160 kDa/59% 100 kDa/16%	The Cu^{2+} absorption capacity of the fungal chitosans appears to be higher than those from *Crustacea* and independent of M of the chitosans from the various sources	Miyoshi et al. (1992)

(*continued*)

TABLE 3.1 (continued)
A Summary of the Major Microbial Sources, Cultivation Conditions, and Type of Fermentation of Several Biotechnological Possibilities to Produce Chitin/Chitosan

Microbial Source	Cultivation Media	Cultivation Conditions, Type of Fermentation	Yield, % Based on Dry Mycelia Weight	Molecular Weight/DA	Description	Reference
Rhizopus sp. *G. butleri* *C. echinulata* *Mucor* sp. *A. glauca*	YPG medium	SmF, Erlenmeyer flasks, 22°C, 48 h, 200 rpm	≈2.05%–5.6% ≈7.7%–93.4% ≈8.0% 5.0%–5.1% ≈6.5%	N/A	*G. butleri* USDB 0201 was found to produce the highest amount of extractable chitosan, followed by *C. echinulata* and *G. butleri* USDB 0428	Tan et al. (1996)
R. oryzae	Basal medium containing 12 g/L D-psicose	SmF, Erlenmeyer flasks 30°C, 70 rpm, 6 days	9.1% chitosan 31.3% AIM	25% for chitosan	Addition of D-psicose in the range 5–12 g/L improved the yield of chitosan but did not cause substantial changes of its physicochemical properties	Yoshihara et al. (2003)
Rhizopus sp. *Mucor* sp. *A. niger*	YPG medium	SmF, Erlenmeyer flasks 30°C, 150 rpm	19%–21% chitosan 25.0% chitosan 11.2% chitosan	N/A	*Mucor* sp. KNO_3 produced the highest amount of extractable chitosan	Nadarajah et al. (2001)
Cunninghamella elegans	Yam bean media and four traditional culture media	SmF, Erlenmeyer flask, 28°C, 150 rpm, 48 h for chitosan and 72 h for chitin	44% chitin 6.6% chitosan	32.5 kDa/93.8% for chitin 27.2 kDa/15% for chitosan	The highest production of biomass and chitin/chitosan can be verified in the yam bean medium	Stamford et al. (2007)
A. niger *R. oryzae* *Z. rouxii* *Lentinus edodes* *P. sajor-caju* *Candida albicans*	PDB medium YMB medium	SmF, Erlenmeyer flask, 30°C, 180 rpm, 15–21 days	11% chitosan 14% chitosan 3.6% chitosan 3.3% chitosan 1.2% chitosan 4.4% chitosan	1.4×10^5/90.0 ± 2.1 6.9×10^4/87.9 ± 2.1 2.7×10^4/85.1 ± 1.1 1.9×10^5/86.5 ± 2.2 1.1×10^5/83.8 ± 0.1 1.1×10^5/83.8 ± 0.8	Chitosan from *Rhizopus* mycelia seems to be highly attractive for medical and agricultural uses	Pochanavanich and Suntornsuk (2002)
Syncephalastrum racemosum	YPG medium	SmF, Erlenmeyer flask, 28°C, 150 rpm	15.2% chitosan	—/28% for chitosan 88.9% glucosamine	Among nine Mucoralean strains (*M. rouxii*, *M. circinelloides*, *C. ramose*, *C. elegans*, etc.), *S. racemosum* exhibited the highest yield of chitosan	Amorim et al. (2003)

A. coerulea	Medium based on yeast extract and glucose	SmF, 2.5 L batch reactor, pH 4.5, 250 rpm, aeration rate 2 vvm, 36 h Continuous reactor (hemostat) at a dilution rate of 0.05 h^{-1}	11.5% chitosan 0.052 g/(L h) chitosan productivity	N/A	Culture conditions were optimized in both batch and continuous reactor systems. The kinetics of chitosan production seems to have a slightly better profile in a continuous reactor	Kim et al. (2001)
A. coerulea etc.	YPG medium	SmF, batch reactor: flask, airlift and stirred tank, pH 5.5, 48 h Continuous reactor	47–50 mg/100 mL 10.9% chitosan, 0.034 g/(L h)	4.5×10^5/6% for chitosan	Supplementation with 0.5 mg of cobalt/100 mL medium showed an increase of approximately 20% in the chitosan yield	Rane and Hoover (1993)
Lentinus edodes	Wheat straw Synthetic medium	SSF, 2 cm particles, plastic bags, 28°C, 60% water content SmF, 25-L stirred tank reactor, 400 rpm, 28°C with airflow	6.18 g chitosan /kg medium after 12 days 120 mg/L chitosan	—/10.1–12.5% for chitosan —/5.5%–12.5% for chitosan	High-quality chitosan was obtained DA ranged from 5% to 13%	Crestini et al. (1996)
G. butleri	Sweet potato pieces	SSF, 3 trays reactor (9.6 L), airflow rate 0.81 min^{-1}, 26°C	1.9%–5.9% depending on extraction method	42–54 kDa/13% for chitosan	The chitosan extraction procedure and the fermentation time are important factors affecting chitosan yield and its molecular weight	Nwe and Stevens (2002)
Auricularia auricula-judae *Pleurotus ostreatus* *Armillaria mellea* *L. edodes*	MEP medium (3% malt extract, 0.5% peptone)	SmF, Erlenmeyer flask, 25°C, stirred, 150 rpm	19.6% chitin, 19.5% glucan 15.3% chitin, 26.7% glucan 11.1% chitin, 16.8% glucan 10.1% chitin, 18% glucan	—/95.1% for chitin —/98.7% for chitin —/92.7% for chitin —/92.0% for chitin	The mycelium of several Basidiomycetes can be considered as a good source of chitin. The characteristics of the fungal chitins were similar to those of commercial chitin	Mario et al. (2008)

Note: YPG media (0.2% yeast extract, 1.0% peptone, and 2.0% glucose); MSM, molasses salt medium (0.2% yeast extract and molasses as carbon source); PDB, potato dextrose broth (20% potato extract and 2% dextrose); YM media (0.3% yeast extract, 0.3% malt extract, 0.5% polypeptone, and 1.0% glucose); AIF, alkali insoluble fraction; DA, degree of acetylation; DDA, degree of deacetylation; DP, degree of polymerization; M, molecular weight; SmF, submerged fermentation; SSF, solid-state fermentation.

3.3.1 Chitin and Chitosan Producer Strains

Microbial chitin and chitosan production has been widely studied particularly by Zygomycetes (Arciadiacono and Kaplan 1992), which are known to contain higher amounts of chitin and chitosan in their cell walls in comparison with other classes of fungi (Campos-Takaki 2005, Shimahara et al. 1989, Zamani et al. 2007). Considerable research has been done by using such strains as *Absidia coerulea* (Davoust and Hansson 1992, Muzzarelli et al. 1994, Niederhofer and Müller 2004), *M. rouxii* (Chatterjee et al. 2005, White et al. 1979, Wu et al. 2005), and *R. oryzae* (Suntornsuk et al. 2002, Yoshihara et al. 2003). These microorganisms can be readily cultured in a minimal medium containing simple nutrients and cell wall chitin and chitosan easily recovered. Tan et al. (1996) reported the chitosan production by 13 strains of Zygomycetes. *Gongronella butleri* USDB 0201 produced the highest amount of extractable chitosan (93.4 mg/200 mL substrate). However, the highest yield of chitosan per unit mycelia mass was obtained from *C. echinulata* (7.14%). In order to determine the fastest growing and greatest chitosan-yielding fungi, Rane and Hoover (1993) also studied different Zygomycetes strains and observed that *Absidia coerulea* was the best chitosan producing strain with a yield of approximately 0.5 g/L of chitosan of medium. Pochanavanich and Suntornsuk (2002) reported the production of chitosan by four filamentous fungi and two yeast strains. *R. oryzae* TISTR3189 was the producer of the highest amounts of chitosan (138 mg/g dry weight). Wu et al. (2005) obtained higher yield of crude chitin from *A. niger* (24.01%) than from *M. rouxii* (13.25%). However, 12.49% of chitosan was obtained from *M. rouxii* dry mycelia and none from *A. niger.*

3.3.2 Cultivation Condition

Microbial chitin and chitosan productions are affected by different factors such as medium composition, temperature, pH, aeration, agitation, size and age of the producing microbial cells, and growth time. Each factor can have various effects on chitin/chitosan production depending on fermentation technique and microorganism under examination.

Göksungur (2004) showed that production of chitosan by fermentation of *R. oryzae* in molasses medium can be increased from 961 to 1109 mg/L by optimization of initial sugar concentration, aeration rate, and agitation speed. In addition, Andrade et al. (2003) investigated the influence of nature and amount of carbon and nitrogen sources represented by the concentrations of D-glucose, L-asparagine, and thiamine, on chitin production by *M. javanicus* by using a statistical design, showing highly significant effects of parameters on the chitin yield. It seems that the most relevant variable for the chitin production was L-asparagine concentration, while time of cultivation did not significantly influence the chitin yield. Similar results obtained on optimization of chitin production by *C. elegans* (IFM 46109) have been reported (Andrade et al. 2000). The highest chitin yield of 28.8% was obtained with a medium containing 60 g/L of D-glucose, 3 g/L of L-asparagine, and 0.008 mg/L of thiamine. Overall, these results are in agreement with Stamford et al. (2007) who reported that chitosan production by *C. elegans* (UCP 542) is strongly dependent on the culture conditions, including cultivation media. Namely, the best yields of chitin and chitosan from *C. elegans* (UCP 542) are obtained using yam bean medium for chitin 400.9 mg/g and chitosan 58.9 mg/g, much higher than those obtained using four different culture media, traditional for Mucorales.

However, the reported data in the literature varied widely among the strains of fungi studied, even between species of the same genus. Thus, Chatterjee et al. (2005) reported that there are no significant differences in the chitosan yield obtained by fermentation of *M. rouxii* on different media. However, there are variations in their polydispersed nature and crystallinity. Chitosan from molasses salt medium was less polydispersed and more crystalline compared to those from yeast extract peptone glucose and potato dextrose broth. Similarly, Nadarajah et al. (2001) determined that there was no significant statistical difference observed in the level of extractable chitosan obtained from the mycelia of *Rhizopus* sp. grown in media containing different glucose concentrations in the range from 1% to 5%.

Although it is very difficult to make general statements on the effects of cultivation media on the chitinous compounds production, several reports show that the supplementation of selected additional medium components could promote chitosan formation. Yoshihara et al. (2003) reported that the D-psicose supplementation (5–12 g/L) in a medium containing a low amount of D-glucose increases the productivity of chitinous substances by *R. oryzae* YPF-61 A, particularly chitosan. Chatterjee et al. (2008) reported that plant growth hormones enhanced the chitosan production by *R. oryzae*. Maximum enhancement was observed with gibberellic acid. Hormones also increase the chitosan quality by increasing of its molecular weight and decreasing polydispersity.

Similarly, urea is the most used nitrogen source to induce chitosan production by filamentous fungi. The production of chitosan by *G. butleri* USDB 0201 was stimulated when urea was added to the solid substrate (Nwe and Stevens 2004). Using solid-state fermentation (SSF) technology, Maghsoodi et al. (2009) showed that urea at a concentration of 6.5 g/kg dry residue increase the chitosan production by *A. niger*, but at higher concentration the yield ceased drastically. In addition, Jaworska and Konieczna (2001) investigated the influence of ferrous ions, manganese ions, cobalt ions, trypsin, and chitin, as individual supplements to the nutrient medium, on chitosan production by the fungus *Absidia orchidis*. Their work suggested that some advantages can be found in producing chitosan by metal-ion regulated liquid medium. Manganese and ferrous ions gave the most significant results while cobalt ions completely inhibited the growth of fungi. It seems that the production of chitosan, which is formed by the complex action of chitin synthase and chitin deacetylase, could be regulated in response to the presence of manganese and ferrous ions in growth medium. On the contrary, there are reports where the supplementation with 0.5 mg of cobalt/100 mL medium showed an increase of approximately 20% in the chitosan yield form *A. coerulea* 14076, suggesting that the requirement for specific metal ions differs from organism to organism (Rane and Hoover 1993).

Factors such as agitation, aeration, pH, and age of microbial cell also strongly influence microbial chitin and chitosan production. Rungsardthong et al. (2006) demonstrated that increase of shaking speed improved both growth and chitosan production by *Abisidia glauca*. Maximal chitosan production of 1.28 g/L at 200 rpm appeared to be twice that of 0.6 g/L when cultivation was carried out at 100 rpm. In addition, it seems that high aeration and agitation may stimulate chitosan production by *A. coerulea* in batch cultivation (Kim et al. 2001). This indicates that mixing has to be sufficient to avoid nutrient and oxygen diffusional limitation providing the fungus to be grown in a suitable morphology to get high yield of chitosan. As to *A. coerulea*, a lower agitation speed appeared to cause the formation of smooth hollow pellets due to oxygen and mass transfer limitations while pellets were not formed at higher speeds.

pH is also an important factor to be considered for chitosan production by several fungi. Rane and Hoover (1993) reported that the pH of the culture influenced the yield and the degree of *N*-acetylation of chitosan from *A. coerulea,* probably due to the optimum pH of the chitin deacetylase. According to Kafetzopoulos et al. (1993), pH 4.5 is optimal for chitin deacetylase activity from *M. rouxii*. The results are in agreement with the study of Kim et al. (2001) who reported that the initial rate of cell growth and chitosan production by *A. coerulea* were significantly improved by controlling the pH at 4.5, showing that pH affected both fungal morphology and chitin deacetylase activity. Likewise, Amorim et al. (2001) reported that higher yields of chitosan were achieved within 24 h of cultivation of *M. racemosus* and *C. elegans* at pH 3.5.

Chitosan production in batch process is mostly growth-associated and apparently no further increase occurs in chitosan yield once the microorganism is in the stationary growth phase. This indicates that during initial growth, chitin (precursor of chitosan) is less crystalline and thus more susceptible to the chitin deacetylase (Davis and Bartniki-Garcia 1984). Therefore, it is not surprising that more chitin in the cell wall appears to be accumulated than chitosan in the late exponential phase (Crestini et al. 1996). The recommended period of fermentation varies from 1 to 21 days depending on the type of culture used for chitin/chitosan production and cultivation conditions (Amorim et al. 2001, Mario et al. 2008). Moreover, there are some conflicting results about growth

rate of *M. rouxii*. The fungus usually reaches their optimal growth within the initial 48–72 h of incubation, whereas maximal chitosan production occurs early in the growth phase (Chatterjee et al. 2005, Tan et al. 1996). For instance, Synowiecki and Al-Khateeb (1997) reported the yields of chitin and chitosan in the mycelia of *M. rouxii* from 2 day old cultures from 8.9% and 7.3%, respectively. Prolonged growth at 28°C (agitation rate 170 rpm) did not significantly influence the available amounts of those polysaccharides. The result is quite different from that reported by Wu et al. (2005) who determined that the total (acetyl)glucosamine content in *M. rouxii* dry biomass increased from 14.2% at day 4 to 20.1% at day 21 by cultivation at 28°C without shaking. This suggests that kinetic parameters of a fungal culture may vary significantly according to culture conditions, particularly agitation, aeration, pH, and others and need to be studied prior to determination of harvest and extraction period.

The profile of microorganism growth and chitin/chitosan production are also investigated using the other Mucorales fungi grown by submerse fermentation. *Cunninghamella elegans* (UCP 542) appeared to reach the late exponential growth phase after only 48 h, producing 24.3 g/L of dry biomass. The best yields of the polysaccharides are obtained with 48 h of culture for chitosan (66 mg/g) and with 72 h for chitin (440 mg/g) (Stamford et al. 2007).

Teng et al. (2001) reported that proteolytic fungal fermentation of shrimp shells is a simple, effective, and inexpensive approach of chitin production from shrimp shells and fungal mycelia. The results suggest that deproteinization and demineralization occurs under those conditions. Sini et al. (2007) reported that *Bacillus subtilis* is an efficient starter culture from fermentation of shrimp shells. About 84% of the protein and 72% of the minerals were removed from the fermented residue at the end of fermentation.

3.3.3 Fermentation Technique

Generally, chitinous compounds have been produced by submerged fermentation (SmF); however, in a few cases, SSF has also been considered promising for chitin and chitosan production (Khalaf 2004, Nwe and Stevens 2002, Rashad et al. 2007). Chitin/chitosan production by SmF technology is usually performed in flask and in a few cases in airlift or conventional stirred tank reactor (Table 3.1). The most common operational mode is batch fermentation. However, continuous fermentation has been successfully applied for chitosan production by *A. coerulea* (Kim et al. 2001). Fermentation by continuous culture in the stirred tank reactor produced the highest amount of chitosan from *A. coerulea* 14076 resulting in an approximate threefold increase in chitosan production as compared to batch culture (Rane and Hoover 1993).

An alternative technology for chitin/chitosan production is SSF. There are conflicting results for the efficacy of SSF for the chitosan production compared to SmF. The growth of the fungus *Gongronella butleri* USDB 0201 under SmF conditions resulted in 1.5–2.5 times higher yield of chitosan compared to SSF using various nitrogen sources (Nwe et al. 2002). By contrast, in another study conducted with *Lentinus edodes*, an opposite pattern was obtained. In this study, the optimal yield of chitosan by SSF was around 6.2 g/kg of fermentation medium, 50 times higher than that obtained with other chitosan production method from fungi (Crestini et al. 1996). These results indicate that the efficiency of the fermentation type used depends on microbial source and must be investigated for each fungi of interest. However, in both cases, SSF seems to be preferred for the production of low acetylation degree chitosan, revealing that this type of fermentation is an interesting option for use on an industrial scale for the production of high-quality chitinous compounds by fungi.

In SSF, several parameters such as particle size, moisture content, incubation time, initial pH, and the amount of nitrogen sources have been shown to be important factors affecting the fungal growth and chitosan production (Nwe and Stevens 2004). An important aspect related to the production of chitosan in SSF has been use of different kinds of laboratory model bioreactors including Erlenmeyer flasks, rotary drum bioreactors, roux bottles, trays, and glass columns to evaluate

the influence of bioreactor design and hydrodynamic conditions on chitin/chitosan production. The basic type of reactor by SSF is the tray reactor that is widely used for fungal fermentation. Giovannozzi-Sermanni et al. (1994) developed efficient innovative solid-state processes for the bioconversion of lignocelluloses by applying new devices and new bioreactor design by choosing the system of tumbling drum system for mixing the material. SSF has shown its potential for chitosan production but it remains yet to be exploited in a major way commercially.

3.4 PHYSICOCHEMICAL PROPERTIES AND COMMERCIAL IMPORTANCE OF MICROBIAL CHITIN AND CHITOSAN

The compositions of microbial chitin resemble those of chitin occurring in other organisms, except for the fact that microbial chitin is usually associated with other polysaccharides (β-glucans and melanins), providing its beneficial properties. In comparison with conventional natural fibers, fungal filaments have the advantage that different kinds of filamentous structures are available, ranging from straight fibers several centimeters in length (sporangiophores) to branched microscopic filaments (mycelium). This chitin-based filamental structure can be applied directly for many biomedical and pharmaceutical applications as wound dressing and carriers of micro-encapsulated drugs, so only relatively cheap chemical treatments are required during processing (Hung et al. 2001, Smelcerovic et al. 2008).

By contrast, the occurrence of chitosan seems to be limited to fungi and its physicochemical properties differ from those obtained by conventional chitin deacetylation process. The quality of chitosan varies with the degree of deacetylation of the *N*-acetyl groups, molecular weight, purity, manufacturing process, color, clarity, consistency, and uniformity. It has been reported that chitosans isolated from Mucorales typically show molecular weight in the range 24.8–450 kDa and degree of acetylation between 6% and 28%, although some exceptions have been cited (see Table 3.1). For example, Miyoshi et al. (1992) reported degrees of *N*-acetylation of 59% and 35% for *M. rouxii* and *C. blakesleeana*, respectively. However, microbial chitosans generally show lower degree of acetylation than those of commercial crabshell chitosans, making them highly attractive for many applications in the food and pharmaceutical industries. Studies on structure–property relationships show that the most effective chitosan for many applications is a medium-low molecular weight chitosan with high density of positive charges, which could be obtained by controlling the fermentation conditions. A range of papers has been published demonstrating the feasibility of obtaining microbial chitin/chitosan with unique, intrinsic properties that render them appealing to serve as an agent for many purposes, foremost as a flocculant in the clarification of waste water (Crestini et al. 1996), as a chelating agent for harmful metals for the detoxification of hazardous waste (Miyoshi et al. 1992), for the clarification of beverages, such as fruit juices and beers (Rungsardthong et al. 2006), as an antimicrobial agent, and for agricultural purposes such as a fungicide in the protection of crops and coating of apples (Wu et al. 2005). In addition, chitosan with a low molecular weight appears to reduce the tensile strength and elongation of the chitosan membrane, increasing its permeability (Rong and Horng 1996). Thus, it could be used as thread or membrane in many medical applications and as a powder in cholesterol absorption (Ikeda et al. 1993).

3.5 CONCLUSIONS AND PERSPECTIVES

The cost of microbial production of chitinous compounds is an important factor in evaluation of their suitability for industrial application. Intensive efforts have been made to optimize the fermentation process for the production of chitin/chitosan with a view to develop economically feasible technologies. The research has been focused on several major aspects: improvement of the yields of chitin/chitosan by screening new microbial sources, and optimization of the fermentation conditions and extraction procedures. An understanding of the structure/function relationships of the

biopolymers and applications should lead to more rational control of their synthesis in industrial settings. Although wild-type microorganisms constitute a valuable source of chitin/chitosan, the advances in molecular biology are allowing the genetic transformation of fungi to obtain strains with high capability for chitinous compounds synthesis.

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4 Enzymatic Production of Chitin from Crustacean Shell Waste

Gyung-Hyun Jo, Ro-Dong Park, and Woo-Jin Jung

CONTENTS

4.1 INTRODUCTION

Chitin in crustacean cuticles is tightly associated with inorganic salts such as calcium carbonate, proteins, and lipids, including pigments. To isolate chitin from crustacean shells, three steps are required, namely, demineralization (DM), deproteinization (DP), and elimination of lipids, including pigments. The traditional method for chitin production was performed through a chemical process using inorganic acids for DM and strong alkali for DP (No et al. 1989, Aye and Stevens 2004). The chemical DM and DP processes have several problems such as they are a source of pollution (Allan et al. 1978), reduce depolymerization and, thus, the chitin quality (Healy et al. 1994, Simpson et al. 1994), the anomerization, and the hydrolytic effects on the chitin structure such as deacetylation (Ng et al. 2000). This process also renders the protein component useless, which otherwise can be used as animal feed and as nutritional additives.

As an alternative to the chemical process, the fermentation process has been studied for decades for various crustacean shells including crab shells (Jung et al. 2006, 2007, Oh et al. 2007, Jo et al. 2008), shrimp waste (Cira et al. 2002, Xu et al. 2008), crayfish exoskeleton (Bautista et al. 2001, Cremades et al. 2001), scampi waste (Zakaria et al. 1998), and prawn waste (Fagbenro 1996, Shirai et al. 1998). On fermentation of crustaceans, two main portions of protein and organic acid salts are recovered for feed, fertilizer, and chemical reagent purposes.

The main objective of all these studies so far was to evaluate the biological process for DM and DP from raw materials of crustaceans. Biological production of chitin using organic acid-producing bacteria and protease-producing bacteria can reduce a source of environmental pollution and the depolymerization of chitin. The main drawback of the biological process is its lower efficiency and quality, it is time-consuming, and thus has a higher cost compared with the chemical process. Yet biological production seems ever promising because of the possible recovery of by-products and that it is less of an environmental burden.

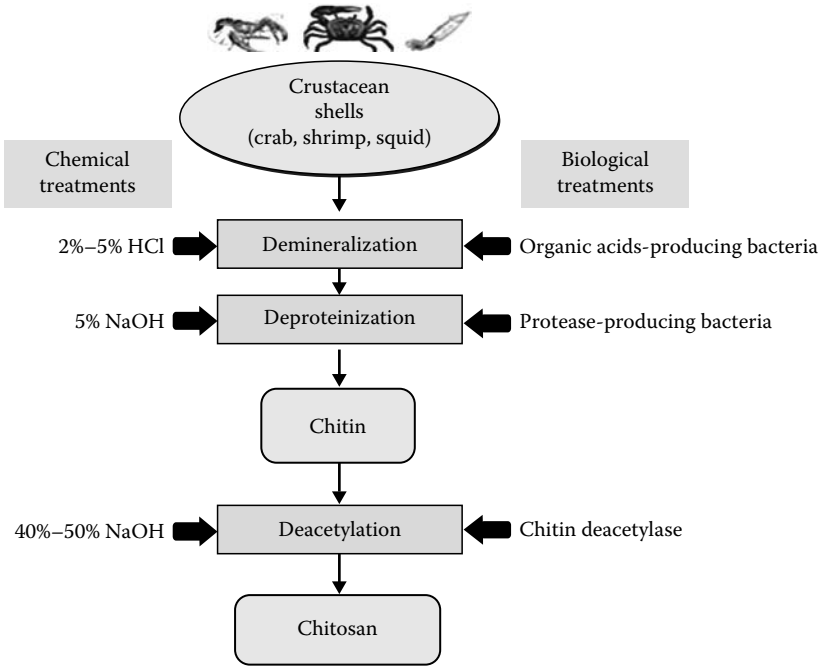

FIGURE 4.1 Chitin and chitosan production by chemical and biological treatments.

This chapter describes the fermentation (or) process of crustacean shells mainly from crab, shrimp, crayfish, scampi, prawn, and squid. Limited literature is available on the isolation of chitin from other sources. Chitin has been extracted from Lucaninade cuticles and Loligo outer walls (Choi et al. 2004), from silkworm pupae, *Psacothea hilaris* (Pascoe), *Agrius convolvuli*, and cockroach shells (Lee et al. 1998), from beetle larvas and the exuviae of silkworm pupae (Zhang et al. 2000), from bumblebees (Majtan et al. 2007) and honeybees (Nemtsev et al. 2004), and fungal mycelia from *Fusarium solani* (Nguyen 2009) and *Aspergillus niger* (Teng et al. 2001) by chemical treatment, using HCl solution for DM and NaOH solution for DP. Furthermore, the yield data is not available in most cases. Thus, we have been much concerned over the fermentations of crustacean shells for the production of chitin. The application and the limitation of the biological approaches are discussed in Figure 4.1.

4.2 BIO-DEMINERALIZATION BY BIOLOGICAL TREATMENTS

The calcium and the protein in the shell waste were dissolved mainly by organic acids and proteases produced by microorganisms (Table 4.1). The efficiency of fermentation depends on factors such as inoculum levels; shell content in medium, shell size; carbon sources such as glucose, sucrose, malt, cassava, molasses, and date juice; initial pH and pH during fermentation; the fermentation type including liquid phase and solid phase cultures; aerobic and anaerobic conditions; and the order in case of successive fermentation consisting of DM and DP processes. Shirai et al. (2001) reported that an increase in the inoculum concentration resulted in improved DM.

For glucose and pH, the addition of glucose facilitates medium conditioning through organic acid production, which suppresses the growth of spoilage microorganisms. Rapid acidification of the medium is very important, especially in industrial scale, to preserve the shell materials fresh, thus minimizing a possible reduction of chitin polymers. Suppression of putrefaction in waste shells was observed at less than pH 5.5. The medium conditioning and ensilation is an efficient method of preservation, allowing the recovery of value-added by-products such as proteins, pigments, and enzymes from crab or shrimp shell wastes (Wang and Chio 1998, Rao and Stevens 2005).

TABLE 4.1
Overview of Biological Treatment of Crustacean Shell Wastes for Chitin Production

Shell Sources	Microorganisms	Products	DM (%)	DP (%)	References
Red crab (*Chionoecetes japonicus*)	*L. paracasei* subsp. *tolerans* KCTC-3074	OA	97.2	52.6	Jung et al. (2006)
	Serratia marcescens FS-3	Protease	94.3	68.9	Jung et al. (2007)
	Pseudomonas aeruginosa F722	OA, protease	92.0	63.0	Oh et al. (2007)
Snow crab (*C. opilio*)	*S. marcescens* FS-3 + Delvolase[a]	Protease	47.0	84.0	Jo et al. (2008)
Squid pen (–)[b]	*Bacillus* sp. TKU004	Protease	—[b]	73.0	Wang et al. (2006)
Shrimp (*Penaeus* spp.)	*Lactobacillus* sp. strain B2	OA	87.6	85.0	Cira et al. (2002)
	L. plantarum 541	OA	86.0	75.0	Rao et al. (2000)
		OA	88.0	83.0	Rao and Stevens (2005) and Kungsuwan et al. (1996)
	Aspergillus niger	Protease	—	62.2	Teng et al. (2001)
	Bacillus licheniformis	Protease	98.8	>99.0	Waldeck et al. (2006)
Shrimp (*Penaeus japonicus*) + Crab (–)	*Pseudomonas maltophilia*	Protease	—	82.0	Wang and Chio (1998)
	B. subtilis CCRC 10029, *P. maltophilia* CCRC 10737, and	Protease	—	88.0	Yang et al. (2000)
	B. subtilis Y-108 *P. aeruginosa* K-187	Protease	—	78.0	Oh et al. (2000)
Shrimp (*Penaeus monodon*)	*Pediococcus acidolactici* CFR2182	OA	72.5	97.9	Bhaskar et al. (2007)
Shrimp (*P. monodon*)	*L. casei* MRS1	OA, protease	99.6	94.7	Xu et al. (2008)
Shrimp (*Crangon crangon*)			99.7	90.8	
Shrimp (*P. semisulcatus*)	*Lactobacillus* sp.	OA	—	—	Khanafari et al. (2008)
Shrimp (*Acetes chinensis*)	*Bacillus* sp. SM98011	Protease	—	—	He et al. (2006)
Shrimp (*Metapenaeopsis dobsoni*)	*B. subtilis* ACC No.121	OA, protease	72.0	84.0	Sini et al. (2007)
Shrimp (*Parapenaeus longirostris*)	*Lactobacillus helveticus*	OA, protease	53	76	Adour et al. (2008)
Shrimp (*Solenocera prominentis*)	*Candida parapsilosis*	Protease	—	75.0	Chen (2001)
Squid pen (*Illex argentines*)	*P. maltophilia*		—	87.0	
Crayfish (*Procambarus clarkii*)	*Lactobacillus pentosus*-4023	OA	90.1	81.5	Bautista et al. (2001)
	L. paracasei A3	OA	97.2		Cremades et al. (2001)
Scampi (*Nephrops norvegicus*)	*L. paracasei* A3	OA	61.0	77.5	Zakaria et al. (1998)

(*continued*)

TABLE 4.1 (continued)
Overview of Biological Treatment of Crustacean Shell Wastes for Chitin Production

Shell Sources	Microorganisms	Products	DM (%)	DP (%)	References
Prawn (–)	*Lactobacillus* sp.	OA	—	77.1	Shirai et al. (1998)
	P. maltophilia LC102	Protease	—	—	Shimahara and Takiguchi (1988)
	L. plantarum, L. salivarius, *Streptococcus faecium*, and *P. acidilactici*	OA, protease	93.8	—	Healy et al. (2003)
Prawn (*Macrobrachium vollenhovenii*)	*L. plantarum*	OA	—	—	Fagbenro (1996)
Shrimp (*C. crangon*)	Alcalase[a]	Protease	94.4	89.0	Synowiecki and Al-Khateeb (2000)
Shrimp (–)	Papaya (*Carica papaya*)[c]	Protease	—	27.0	Indra Jasmine et al. (2006)

Note: OA, organic acid; DM, demineralization; DP, deproteinization.
[a] Commercial enzymes.
[(—)b] Not mentioned.
[c] Plant enzymes.

Ensilation produces a change in the waste from semisolid to liquid in the case of shrimp shells within 2–3 days with pH reduction. The liquefaction of the shell waste occurs mainly by proteolytic enzymes produced by starter microorganisms, gut bacteria present in the intestinal system of biomaterials, or proteases present in the biowaste. The separated liquor represents 60%–70% of the silage and is composed of proteins and lipids. More than 85% of the chitin present in the substrate remains in the sediment (Shirai et al. 1998). Lactic acid-producing bacilli were mostly adopted for the acidification and decalcification processes. The production of organic acids by the lactic acid bacterium *L. plantarum* decreased the pH in aqueous liquid medium and made the environment selective against spoilage microorganisms (Hong et al. 1999). Over the first 48 h of scampi waste fermentation in 10% glucose and 10% inoculums (*L. paracasei* strain A3), the pH of the liquor achieved a minimum value of 5.0 (Zakaria et al. 1998).

Fermentation using *Lactobacillus pentosus* 4023 was applied for DM of crayfish shells and resulted in DP 90.1%, a rather promising efficiency (Bautista et al. 2001). Efficiency of lactic acid fermentation for DM of crab waste shells with *L. paracasei* KCTC-3074 was compared with chemical treatments such as 2 N HCl, 0.1 M EDTA, and 0%–10% lactic acid (Jung et al. 2005). The DM rates were 75%–82%, depending on the amount of inoculums, lower than that of the chemical process. During the process, DP also occurred with rather high rates. For instance, in DM for shrimp (*Penaeus* spp.) wastes using *Lactobacillus* sp. B2, a DM rate of 85% was observed concomitantly with a DP rate of 87.6% for a 6 day culture (Cira et al. 2002). In the process, a negative correlation between DM and pH in culture broth but a positive one between DM and the total titratable acidity were observed (Jung et al. 2005).

As expected, recovery of the calcium salt of organic acids, mainly calcium lactate, from the culture medium needs attention, but little has been reported. Calcium lactate is formed during fermentation and precipitates, which can be removed by washing. The resulting organic salts from the DM process could be used as de- and anti-icing agents and/or preservatives.

4.3 BIO-DEPROTEINIZATION BY BIOLOGICAL TREATMENTS

4.3.1 Deproteinization by Protease-Producing Bacteria

In bacterial fermentation for chitin and chitosan production, the most often applied strains are *Lactobacillus* sp., *Bacillus* sp., *Pseudomonas* sp., and *S. marcescens*. The microbial DP process is little efficient, ranging between 50% and 85% DP rate depending on materials, used microorganism, fermentation type, and time. Rao et al. (2000) cultured shrimp biowaste with *L. plantarum* and achieved 75% DP. Bautista et al. (2001) achieved 81.5% DP from crayfish using *Lactobacillus pentosus* 4023. Fermentation of crab shell wastes with 10% *S. marcescens* FS-3 inoculum resulted in DP of 84% and DM of 47% at 7 days culture (Jo et al. 2008). Squid pen for the preparation of β-chitin were deproteinized by 73% for 3 days with *Bacillus* sp. TKU004 (Wang et al. 2006). Also, the shrimp shells were deproteinized by 75% and 87% at 30°C for 6 days with *Candida parapsilosis* and *Pseudomonas maltophilia*, respectively (Chen 2001).

P. aeruginosa K-187 is a producer of protease, chitinase, and lysozyme. In liquid phase fermentation of shrimp and crab shell powder (SCSP), shrimp shells, and shrimp heads, DP rates were 55%, 48%, and 61% at 7, 5, and 5 days after fermentation, respectively. In solid phase culture, DP of SCSP, acid-treated SCSP, shrimp shells, and shrimp heads was 68%, 46%, 82%, and 81% at 10, 10, 5, and 5 days after fermentation, respectively (Wang and Chio 1998).

In this process, it was found that crab shell content affected on the DP rate. When fermented with *S. marcescens* FS-3, 80% DP rate was recorded in 5%–10% shell content where the entire shell was submerged in the culture medium (solid to liquid ratios, 1:10–20 w/v), but more than 55% DP in 20%–30% shell content, where semisolid or solid phase fermentation occurred (solid to liquid ratios, 1:3.3–5 w/v) (Jo et al. 2008). DM also declined from 52.1% to 18.9% according to the shell content, showing an efficiency of submerged culture over the solid phase fermentation and a limitation of increasing shell content in the culture medium. As other microbial source, fungi also have been tried for the fermentation. *Aspergillus niger* facilitated the DP of shrimp-shell powder and the release of hydrolyzed proteins (Teng et al. 2001).

4.3.2 Deproteinization by Enzymes

A few studies on use of proteolytic enzymes for the DP of crustacean wastes have been reported. Tuna proteinase, papain, and a bacterial proteinase have been used for DP (Broussignac 1968, Indra Jasmine et al. 2006). The residual protein associated with the chitin after enzyme treatment was about 5% (Takeda and Abe 1962, Takeda and Katsuura 1964). Gagne and Simpson (1993) also showed that the residual protein levels in the shrimp waste after the DP were 1.3% and 2.8% for chymotrypsin- and papain-treated samples, respectively. High enzyme to waste ratio (*E/W*) was needed for maximum DP. The typical values of *E/W* ratio were 0.7% and 1.0% (w/w) for chymotrypsin and papain, respectively. Jo et al. (2008) compared the DP efficiencies of commercial enzymes such as Delvolase®, Cytolase PCL5®, Econase CEPi®, Econase MP 1000®, Maxazme™ NNP®, and Ccllupulin MG®, and found out that Delvolase showed the highest DP activity for crab shell waste. When treated with 1% Delvolase, the DP rate reached to about 85% within 1 day and increased up to 90% thereafter. The commercial enzyme Delvolase was most rapid and effective in DP of the crab shells, but no complete removal of the residual protein associated with the chitin.

There were a few reports comparing the DP effects between microbes and enzymes. Bustos and Michael (1994) have compared the effects of microbial and enzymatic DP. A maximum value of 82% in DP was achieved with *P. maltophilia* after 6 days of incubation, but no more than 64% in DP was achieved by using a purified microbial protease under the same condition. Rao et al. (2001) applied the combination of papain and GBW protease as commercial enzymes on the DP of shrimp wastes and found that removal rates of protein in the waste with enzymes were low.

4.4 COMBINATION OF BIO-DEMINERALIZATION AND BIO-DEPROTEINIZATION

Some bacteria such as *Lactobacillus* sp. produce protease as well as organic acid on bio-fermentation. These strains are ideal for simple one-step fermentation to extract chitin from the biomaterials. By a microorganism, DM and DP occur concomitantly in a batch culture. For shrimp heads, 83% DP and of 88% DM and for shrimp shells of 66% DP and 63% DM were achieved with *L. plantarum* 541 (Rao and Stevens 2005). The fermentation of shrimp biowaste has been optimized at pH 6.0 using 10% *L. plantarum* inoculum and 5% glucose, and pH 6.0 adjusted with acetic acid, which resulted in simultaneous 86% DM and 75% DP of the solid fraction (Rao et al. 2000). Moreover, DP of 97.9% and DM of 72.5% were achieved at 72 h after fermentation of shrimp (*Penaeus monodon*) biowaste using *Pediococcus acidolactici* CFR2182 (Bhaskar et al. 2007).

Healy et al. (2003) once tried cofermentation with lactic acid-producing bacteria (*L. plantarum* and *L. salivarius*) and protease-producing bacteria (*Streptococcus faecium* and *P. acidilactici*) to produce chitin from prawn shell (*Nephrops norvegicus*) in a one-batch culture under anaerobic condition for 7 days. After that, Jung et al. (2006) introduced *L. paracasei* KCTC-3074 and *S. marcescens* FS-3 for extraction of chitin from crab shells. However, in the cofermentation, DM was 97.2% but DP was 52.6%, much lower than expected, for 7 day fermentation. This result suggested the initial drastic pH drops in cofermentation of two strains mainly due to a lot of production of organic acid from *L. paracasei* KCTC-3074. Therefore, the proliferation of *S. marcescens* FS-3 should be affected by the severe variation of pH, thus resulting in less secretion of proteases and less DP. In the cofermentation with at least two different strains for DM and DP together in a one-batch culture, this kind of problem would be always encountered. Thus, the discovery and characterization of novel microorganisms that proliferate and secrete organic acids and proteases will be necessary in the near future.

A successive two-step fermentation to produce the final product, chitin from biomaterials has been also challenged. One can use one microorganism that produces both organic acids and proteases or two different ones, that is, one is organic acid producer and the other is protease producer. Often, acidification and DM process come first for a stable waste ensilation. Jung et al. (2007) tried a successive two-step fermentation with lactic acid-producing *L. paracasei* KCTC-3074 and protease-producing *S. marcescens* FS-3 with red crab shells, and 94.3% DM and 68.9% DP were obtained. This data suggested that DP was not still satisfactory and needed more treatments (Jung et al. 2007). Recently, exceptional protocol has been tried to improve DP efficiency with a single strain (Xu et al. 2008). They controlled oxygen content anaerobically during two-stage fermentation of deproteination and decalcification using homofermentative *Lactobacillus casei* MRS1. Shrimp (*Penaeus monodon*) and shrimp shells (*Crangon crangon*) were treated by this protocol, resulting in 99.6% and 99.7% DM, and 97.4% and 90.8% DP for *P. monodon* and *C. crangon* shells at 4 days after fermentation, respectively.

4.5 OPPORTUNITY FOR THE BIOLOGICAL TREATMENTS

Very recently, much progress has been reported in extraction of chitin and chitosan from *Penaeus semisulcatus* waste (Khanafari et al. 2008). When compared with chemical method (2% NaOH for DP and 10% acetic acid for DM), the microbial method using *Lactobacillus* spp. resulted in a better extraction of chitin, providing the effectiveness of the lactic acid fermentation as an alternative to chemical treatment.

As mentioned earlier, the minerals and proteins are not completely removed by the biological treatment. The residual protein and ash could be removed from the fermented materials by mild chemical treatment. Sini et al. (2007) treated the materials after fermentation (84% DP and 72% DM of shrimp shells) with mild acid (0.8 N HCl) and alkali (0.6 N NaOH) to obtain characteristic chitin (*N*-acetylation 84.4%, protein 0.81%, and ash 0.85%). The optimum concentrations of acid and alkali required depend on the residual protein and ash content, solid to liquid ratio, the type of raw materials, and treatment time.

It is worthy to mention that different from the organic acid salt in DM process, the by-product protein concentrate has received much attention from researchers to recover for animal feed supplement and for the preparation of protein hydrolyzates enriched in essential amino acids for human nutrition. Cremades et al. (2001) obtained carotenoproteins and chitin from clawfish by a combined process based on flotation-sedimentation and in situ semisolid lactic acid fermentation. During fermentation of the shrimp, oligopeptides were produced as an additive by-product using *Bacillus* sp. SM98011 (He et al. 2006).

Since crab and shrimp proteins in powder form for human consumption have a value at least equal to that of chitin, designing a chitin extraction procedure that results in the isolation of the protein is attractive. This would reduce the environmental problem of the high biological oxygen demand (BOD) of the effluent. Aye and Stevens (2004) reported that physical treatment, such as drying, grinding, and sieving, were enough for the recovery of 50% shrimp protein as a dry powder, and pretreatment of shells with shearing in acidified water resulted in 60% removal of the protein and a reduction of the mineral content without a decrease in chitin yield.

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5 Continuous Production of Chitooligosaccharides by Enzymatic Hydrolysis

Se-Kwon Kim and Jae-Young Je

CONTENTS

5.1 INTRODUCTION

Since the discovery of chitosan in the late 1850s, many studies were performed to understand its fundamental physicochemical properties. Chitosan, a nontoxic biopolymer, is a partially deacetylated polymer of *N*-acetyl glucosamine (GlcNAc), which is obtained after alkaline deacetylation of the chitin, derived from the exoskeletons of crustaceans and arthropods. Chitosan is a natural, less toxic, biodegradable, biocompatible compound, and its versatile chemical and physical properties have received much attention to a broad range of commercial applications such as food (Shahidi and Synowiecki 1991), cosmetics (Majeti and Kumar 2000), biomedicine (Felt et al. 1998), agriculture (Yamada et al. 1993), environmental protection (Peniche-covas et al. 1987), and wastewater management (Jeuniaux 1986). Further, physicochemical properties of chitosan especially encourage its potential to use as a bioactive material (Kurita 1998). Although chitosan is known to have important functional properties in many areas, its application for industry is restricted because of poor solubility and absorption through the human intestine. Unlike chitosan, chitooligosaccharides (COSs), the hydrolyzed products of chitosan are readily soluble in water due to their shorter chain lengths and free amino groups in D-glucosamine (GlcN) units (Jeon et al. 2000). The low viscosity and greater solubility of COSs at neutral pH have attracted the interest of many researchers to utilize chitosan in its oligosaccharides form. Furthermore, COSs also have versatile biological activities such as antitumor (Suzuki et al. 1986), immune-stimulating effect (Jeon and Kim 2001), enhancement of protective effects against infections associated with some pathogens in mice (Yamada et al. 1993), antifungal (Hirano and Nagao 1989), and antimicrobial activities (Jeon et al. 2001). Therefore, over the past decade, researchers in Asia, Europe, and North America have tested COSs in biomedical applications. Chitosan-based researchers have also been focused on the food and nutrition areas, including the development of edible films and coatings to preserve the quality and texture of foods.

Chemical and enzymatic methods are the widely used COS production approaches and among them chemical hydrolysis is used more commonly in the industrial-scale production. However, chemical hydrolysis has some drawbacks to be commercialized, due to the development of some toxic compounds, higher risk associated with the environmental pollution, and

lower production yields. Therefore, lack of proper technology for the large-scale manufacturing of COS with desired molecular weights made it difficult for human use in the past years. The enzymatic processes are generally carried out in batch reactors and are preferable over chemical methods. However, the higher cost associated with hydrolytic enzymes reduce the application of enzymatic methods. Therefore, reuse of enzymes is recommended and sequential development is achieved in continuous production of COS in large scale. This chapter provides an overview of the development of novel methods for the continuous production of COSs by enzymatic hydrolysis.

5.2 COS PREPARATION METHODS

Chitosan is the starting materials of COS, which is a derived form with a cationic nature from chitin by *N*-deacetylation under alkaline condition. Chitosan can also be cleaved by hydrolyzing agents due to the presence of rather unstable glycosidic bonds similar to all polysaccharides. Cleavage of glycosidic bonds by different methods leads production of COS with variation in the degree of polymerization (DP) as well as number and sequence of GlcN and GlcNAc. Some of these methods include, acid hydrolysis (Il'ina and Varlamov 2004), enzymatic hydrolysis (Kuroiwa et al. 2002), oxidative degradation (Shirui et al. 2004), ultrasonic degradation (Chen and Chen 2000), chemoenzymatic (Akiyama et al. 1995), and recombinant approaches (Samain et al. 1997).

For large-scale production of COS, acid hydrolysis is commonly used to cleave glycosidic linkages of chitosan. However, chemical hydrolysis results low yields of COS and a larger amount of monomeric D-GlcN units (Uchida et al. 1989). Therefore, COS prepared by industrial-scale acid hydrolytic methods are generally not considered to serve as bioactive materials due to the possibility of contamination of toxic chemical compounds. As a result, enzymatic hydrolysis of chitosan has been proposed as a preferred method for the production of bioactive COS during past few decades.

5.3 ENZYMATIC PREPARATION OF COS

Chitosan is generally susceptible to a number of different enzymes and that indicates its broad substrate specificity (Aiba 1994). Up-to-date, a range of chitosanase has been reported from different microorganisms including fungi and bacteria, as well as some other common carbohydrases and proteases are also have proven their hydrolytic ability on chitosan to produce COS with various molecular weights (Aiba 1994, Kim et al. 1998, Lee et al. 1996). However, production performance of chitosanases derived from microbes differ in their catalytic action because differentially deacetylated chitosans have four different types of randomly distributed glycosidic bonds in their structures, and the enzymatic activity is mainly dependent on degree of deacetylation (Aiba 1994). However, it has been generally observed that chitosanases obtained from microbes produce relatively a higher yield of COS compared to chitosanases from other sources. Even though, microbial chitosanases have shown to have excellent performances in COS production, they are too expensive to be utilized in large-scale industrial application. Therefore, other commercial enzymes are utilized under specific conditions to produce COS with relatively low cost (Zhang et al. 1999).

Initially, enzymatic hydrolysis was carried out in batch reactors (Figure 5.1), where chitosanase was mixed with its substrate, and allowed to break down glycosidic bonds of chitosan under optimum conditions (Izume and Ohtakara 1987). Optimum conditions of chitosanases derived from *Bacillus pumilus* BN-262 and *Bacillus* sp. No. 7-M were documented with similar hydrolyzing conditions for producing COS in batch reactor (Jeon and Kim 2000a, Varum et al. 1996). However, this batch system had some disadvantages such as low yields and higher cost associated with the use of large quantities of expensive chitosanase. Later, it was found that a number of different enzymes can also be used for the hydrolysis of chitosan. For example, lysozyme and chitinase can act on partially *N*-acetylated

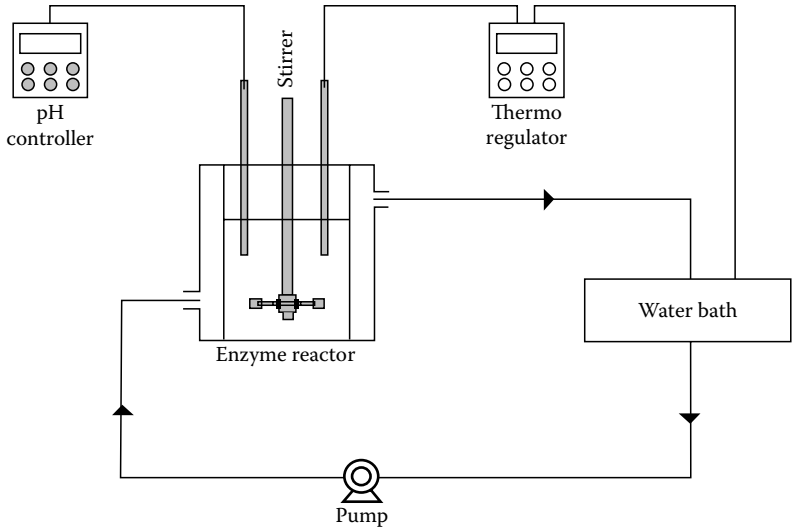

FIGURE 5.1 Typical schematic diagram of a batch reactor for the hydrolysis of chitosan using free enzyme. (From Kim, S.K. and Rajapakse, N., *Carbohydr. Polym.*, 62, 357, 2005. With permission)

chitosan by recognizing GlcNAc residues in the chitosan sequence (Aiba 1994). However, the main drawback of this enzymatic hydrolysis was inefficient production of desirable chain lengths of COSs, which were not applicable for large-scale production of bioactive COSs.

In order to overcome these problems, a new method for producing COSs with higher DP by means of a column reactor packed with an immobilized chitosanase was developed (Jeon et al. 1998). The highest enzymatic activity was observed while the chitosanase immobilized on chitin than other carriers. However, as observed in many related studies, immobilization of chitosanase could also not provide promising results as expected. The immobilized chitosanase showed a lower affinity and lower reaction rate than the free chitosanase; thereby in this method the yield of COS was lower. Therefore, an enzyme reactor system together with an ultrafiltration (UF) membrane reactor was developed to produce COS with relatively a higher DP (Jeon and Kim 2000a). This system could hydrolyze substrate that is equivalent to 11 batches used in the batch reactor with the same amount of enzyme, and enabled effective production of relatively large COS that have shown interesting biological activities in other studies. The most important factor in the UF reactor system was the control of permeation rate that determines the components of the resultant oligosaccharides. However, UF membrane method did not allow continuous production of COS due to the increased transmembrane pressure during the reaction. This was due to high viscosity of chitosan solution and fouling of membrane by accumulated substrate. Therefore, reduction of viscosity of chitosan prior to treatment in the UF membrane system was a requisite for a more effective continuous production system.

The continuous production of COS was found to be feasible with combination of a column reactor packed with immobilized chitosanase and UF membrane system, and this system was developed by Jeon and Kim (2000b), named as dual reactor system (Figure 5.2). In this system, production of COSs may be performed in two steps. In the first step, chitosan is partially hydrolyzed by the immobilized chitosanase prepacked in the column reactor and the product is supplied to the UF membrane system for the production of COS. As expected, the viscosity of partially hydrolyzed chitosan was low thereby it does not create fouling problems under controlled conditions, and continuous production of COSs was achieved. This method ensures a greater productivity per unit enzyme, ability to control molecular weight distribution and more efficient continuous production process compared to those of conventional methods. Therefore, this method is commonly used to produce different molecular size COSs to study their bioactivities.

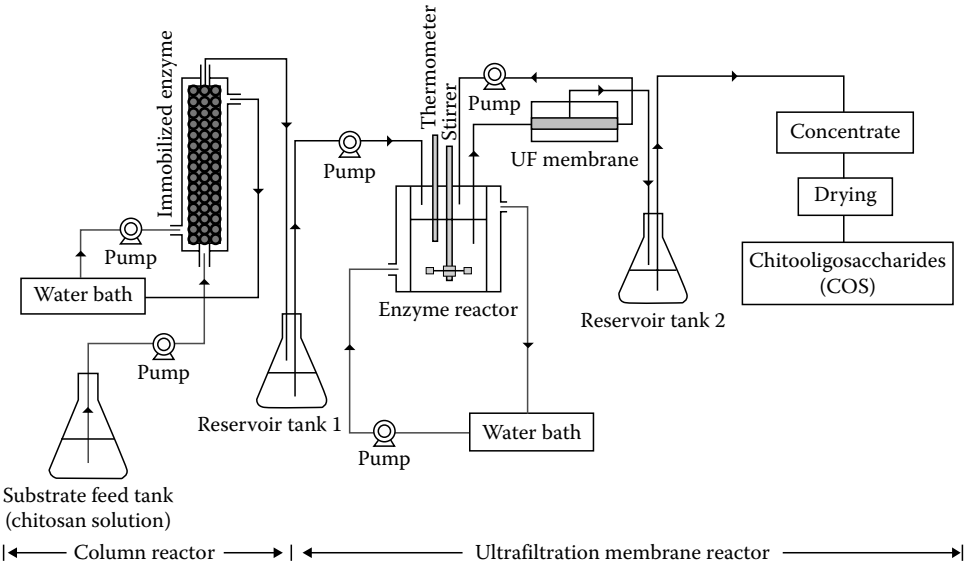

FIGURE 5.2 Schematic diagram of the dual reactor system developed for continuous production of COSs. (From Jeon, Y.J. and Kim, S.K., *Proc. Biochem*., 35, 623, 2000b. With permission.)

5.4 CONCLUSION

COSs derived from chitosan have become much attention due to their versatile biological activities. Although COSs can be produced using different methods, enzymatic hydrolysis of chitosan is the most reliable and effective method to obtain bioactive COSs with higher purity, used for human consumption.

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6 Biosynthesis of Cellulose–Chitosan Composite

Muenduen Phisalaphong, Nirun Jatupaiboon, and Jeerun Kingkaew

CONTENTS

6.1 INTRODUCTION

Cellulose and chitin are recognized as the first and the second most myriad of polysaccharides, respectively. Generally, chitin's resources are crustaceans like crab, prawn, lobster, and shrimp shell waste. Chitin is also widely distributed in marine-like invertebrates, insects, fungi, and yeast. Chitin is a long-chain polysaccharide composed of linear β-1,4-linked units of *N*-acetyl-D-glucosoamine (Rinaudo 2006). Chitosan is a biopolymer derived from chitin by deacetylation of acetyl groups ($CO–CH_3$) (Figure 6.1).

Both chitin and chitosan have unique properties, including polyoxysalt formation, ability to form films, chelate metal ions, and optical structural characteristics (Ravi Kumar 2000). Chitin is highly crystalline, hydrophobic, and insoluble; however, chitosan can dissolve in dilute acids such as acetic acid, formic acid, and so on. The applications of chitosan have been found in many fields, for instance, wastewater treatment, photography, cosmetics, ophthalmology, agriculture, paper finishing, drug-delivery systems, and food industry (Ravi Kumar 2000). Chitosan has good chemical and biological qualities that can be used in many medical applications. Due to the similarity of its structural to glycosamino glycans, it was considered to be used for developing substratum for skin replacement (Sandford and Stinnes 1991). Chitosan could be used to inhibit fibroplasia in wound healing, and to promote both tissue growth and differentiation in tissue culture (Muzzarelli et al. 1999). It has been reported as a good material for gene delivery, tissue engineering, and wound healing (Rigby et al. 1997, Ciechańska 2004, Rajendran and Anand 2006, Petrulyte 2008).

Cellulose is a linear polysaccharide composed of D-glucose units jointed together by β-l,4-glucosidic bonds as shown in Figure 6.1 (Brown et al. 1983). It is because of its long molecules; cellulose is insoluble or does not dissolve easily in water. In general, the advantages of cellulose include high specific strength and good thermal stability. The cellulose's structure can be defined traditionally as two major types: cellulose I and cellulose II. Both of them have a difference in the polarity of the cellulose chains. The backbone conformations of the chains themselves are essentially identical. Cellulose I has parallel chains, whereas cellulose II has alternating antiparallel chains (Gardner and Blackwell 1974).

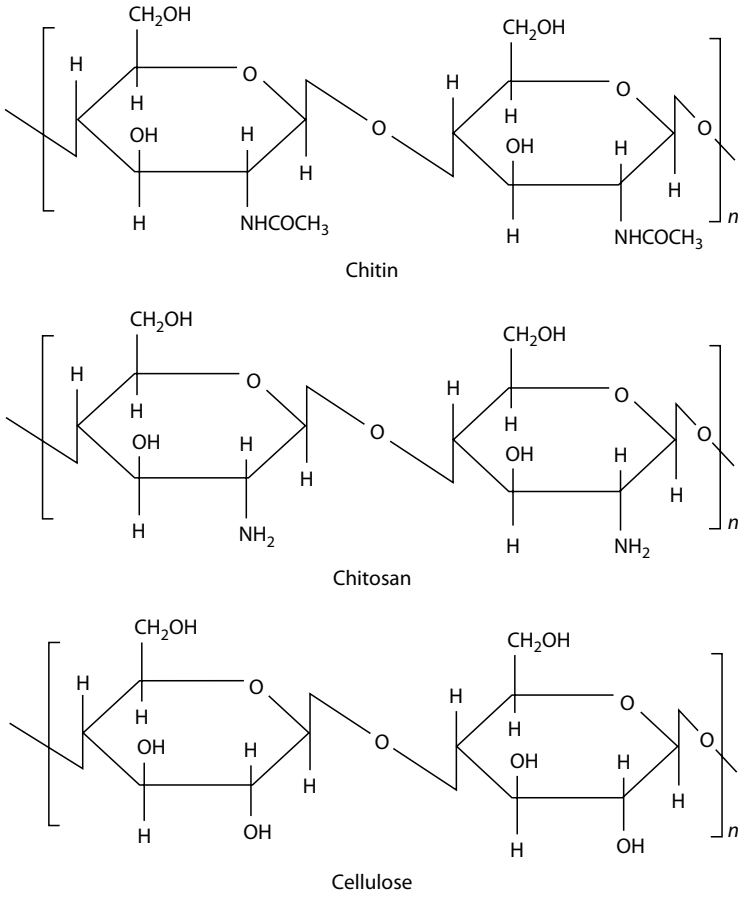

FIGURE 6.1 Chemical structure of chitin, chitosan, and cellulose.

Over the past 40 years, there has been a heightened interest in the biosynthesis of cellulose by *Acetobacter* bacteria (Dimaguil-Saturnino 1967, Brown et al. 1976, Brown 1989, Czaja et al. 2006). Bacterial cellulose (BC) is obtained from the polymerization process into long microfibrils outside the cell wall. BC originates as a white gelatinous pellicle composed of cellulose nanofibrils of 3–8 nm diameters on the surface of the liquid medium in a static culture. Its crystallographic form of the microfibrils containing parallel extended glucan chains is designed as cellulose I (Ross et al. 1991). BC has the same chemical structure of plant cellulose; however, the size of BC fibrils is about 100 times smaller than that of plant cellulose (Cannon and Anderson 1991, Czaja et al. 2006, Phisalaphong et al. 2008). This nanostructure results in a large surface area. Consequently, BC has remarkable water-holding capacity. It is extremely hydrophilic, absorbing 60–700 times its weight in water (Phisalaphong et al. 2008). In addition, BC has outstanding mechanical properties, high crystallinity, and highly pure fiber network.

There have been a wide range of applications of BC in numerous areas. For example, it has been used in acoustic diaphragms for audio speakers to create a sound transducing membrane (Yamanaka and Watanabe 1994, Wan 2006). Adding BC to paper pulp resulted in creating a stronger paper (Yamanaka and Watanabe 1994). In the food industry, BC is used as a food additive for a chocolate drink in place of xanthan gum (Okiyama et al. 1993). As a dialysis membrane, BC shows a significantly higher permeation rate and a greater molecular weight cutoff relative to a commercial dialysis membrane from regenerated plant cellulose (Shibazaki et al. 1993). BC's unique properties lead

to its proposed uses in biomedical applications. The applications include artificial skin for burn and skin injuries (Fontana et al. 1990, Jonas and Farah 1998), the replacement of blood vessels (Klemm et al. 2001), scaffold for tissue engineering of cartilage (Svensson et al. 2005), and wound dressing (Czaja et al. 2006). BC is an interesting material for wound dressing since it is able to maintain wound in a wet condition, inexpensive, lightweight, flexible, and impermeable to microorganisms (Czaja et al. 2006).

In order to develop BC with chitinous properties, chemical structure of BC is modified by the supplement of chitin, chitosan, or its derivative into the culture medium during biosynthesis by *Acetobacter* bacteria. Since the chemical structure of chitosan backbone is very similar to that of cellulose (Figure 6.1), it was expected that chitosan could be miscible with cellulose and incorporated into the cellulose chain. By this modification, it might improve the chemical, physical, mechanical, and biological properties of the developed composite. It has been reported that with the supplement of chitosan and chitosan derivatives in the culture medium, glucosamine and *N*-acetylglucosamine units could be incorporated in the cellulose chain (Ogawa and Tokura 1992, Ogawa et al. 1992, Ciechańska et al. 1998). In a series of papers published in 1992–1997, it was demonstrated that *N*-acetylglucosamine (GlcNAC) could be incorporated to BC by the incubation of *Acetobacter* in liquid medium with GlcNAc as a carbon source or as a solid medium (Ogawa and Tokura 1992, Ogawa et al. 1992, Shirai et al. 1994, 1997). The results from amino acid analysis showed that the maximum content of GlcNAc residues in BC was 4.5 mol% in cellulose (Shirai et al. 1994). It was concluded that the amount of degraded chitosan segments that was incorporated into polysaccharide chains was dependent on the amount and type of chitosan including the culture condition. HPLC results indicated that the P-chitins in the culture medium were depolymerized to monomeric and oligomeric P-chitins during the incubation and were then utilized by *Acetobacter* as a carbon source. It was suggested that GlcNAc 6-P might isomerize to GlcNAc 1-P and be incorporated into the polysaccharide chain by the same metabolic pathway of glucose (Shirai et al. 1997). From FTIR spectrum, amide bands were observed showing the presence of amino groups in the modified BC composite (Phisalaphong and Jatupaiboon 2008). Intermolecular hydrogen bonding interaction between cellulose and amino group of chitosan was detected (Phisalaphong and Jatupaiboon 2008). The incorporation of chitosan is later found resulting in many improved characteristics of BC–chitosan composite (Ciechańska 2004, Phisalaphong and Jatupaiboon 2008, Kingkaew et al. 2010). This chapter emphasizes the biosynthesis and characteristics of cellulose–chitosan composite by static incubation of *A. xylinum* in liquid medium containing chitosan. A review of the literature is also provided.

6.2 BIOSYNTHESIS

6.2.1 Microorganisms

Some kinds of bacteria such as *Acetobacter*, *Rhizobium*, *Agrobacter* can produce extracellular cellulose. However, commercial applications of BC are mostly produced by *A. xylinum*. *A. xylinum*, a gram-negative bacterium, has the ability to synthesize large quantity of high-quality cellulose in the form of flat, twisting ribbons of microfibrillar bundles (Czaja et al. 2006). *Acetobacter* bacteria have also been used for the production of vinegar, gluconic acid, and ketogluconic acid as well.

6.2.2 Culture Media and Method

The Hertrin–Schramm medium is a standard medium used for *Acetobactor* cultivation and formation of BC (Hestrin and Schramm 1954, Shirai et al. 1997). Glucose or sucrose is usually employed

as a carbon source. In many tropical countries, coconut and pineapple juices as wastes from agro-industries are used as commercial raw materials for BC production. Nata de coco and nata de pina are well-known products of BC synthesized by *A. xylinum* in coconut and pineapple juice medium, respectively. However, it was reported that coconut juice was a better substrate for the growth of *Acetobacter* bacteria than pineapple juice (Kongruang 2008). Nonetheless, other simple carbohydrates, alcohols, or polyalcohols can be considered as carbon sources (Brown 1991). During the cellulose biosynthesis, many carbon compounds of the culture medium are utilized by the bacteria, then polymerized into single, linear β-1,4-glucan chains and finally secreted outside the cells through a linear row of pores located on their outer membrane. Bacteria build BC and confine themselves in it to protect themselves from enemies and heavy-metal ions while nutrients can be supplied by diffusion (Williams and Cannon 1989).

A method of producing biosynthetic cellulose–chitosan composite by the supplement of low molecular weight chitosan has been reported (Phisalaphong and Jatupaiboon 2008). BC–chitosan pellicle was developed in culture medium composed of coconut juice supplemented with 5.0% sucrose, 0.5% ammonium sulfate [$(NH_4)_2SO_4$], 1.0% acetic acid, and 0.75% (w/v) chitosan of MW 30,000–80,000 with 85%–90% degree of deacetylation. Mostly, coconut juice contains about 2%–3% of sucrose varied from the location of the plantation, the maturity, and the plant variety. Therefore, the total amount of initial sugar concentration for the cultivation was about 7%–8% (w/v). The sterilization of the culture medium was carried out at 110°C–115°C for about 5 min. The inoculum level introduced into a production flask was 5% by volume. The BC–chitosan biosynthesis was carried out at 30°C for 8–14 days. The other methods to produce BC–chitosan composites were previously reported by using the modified Hestrin–Schramm medium supplemented with *N*-acetylglucosamine (Ogawa and Tokura 1992, Ogawa et al. 1992), with water-soluble P-chitins (Shirai et al. 1997) or with chitosan acetate and chitosan lactate (Ciechańska et al. 1998, Ciechańska 2004).

6.2.3 Formation and Growth Kinetics

During the cultivation, BC is produced in the form of a thin, leather-like white pellicle at the air–liquid interface of the culture. BC is a growth-associated product (Kongruang 2008); therefore, the cellulose formation is proportional related to the cell growth. *Acetobacter* is an aerobic bacteria; the buoyancy of its cellulose could provide an aerobic environment for the cells. It has been previously reported that in static cultivation, the rates of cell growth, cellulose formation, and the consumption of glucose are controlled by the diffusion of atmospheric oxygen (Budhionoa et al. 1999). The liquid–air interface area and the depth of liquid medium are therefore important parameters for bacteria cellulose biosynthesis. The increment of medium volume by increasing the depth of liquid medium was less effective for cellulose production than increasing the surface area (Phunsri et al. 2003). The pH of fermentation medium also strongly affects the growth and cellulose formation of *Acetobacter* sp. The optimum pH was reported at 4.0–6.0 (Masaoka et al. 1993, Phunsri et al. 2003). Other control parameters for growth and cellulose formation are bacterial strains, carbon and nitrogen sources, and temperature.

The appearance of the pellicle of cellulose–chitosan is shown in Figure 6.2. The cell growth and BC production profiles of *A. xylinum* in the coconut juice medium modified with 0.75% chitosan of MW 30,000 and 80,000 compared with those in the unmodified coconut juice medium are shown in Figures 6.3 and 6.4.

In a static condition, typical BC formation rate was $1.0–2.4 \times 10^{-3}$ $g_{(dry\ wt)}$ cm^{-2} day^{-1} (Masaoka et al. 1993, Jonas and Farah 1998, Sanchavanakit et al. 2006). The BC productions of *A. xylinum* (AGR 60) in the modified coconut juice medium with chitosan of MW 30,000 and 80,000 were at 0.43×10^{-3} and 0.37×10^{-3} $g_{(dry\ wt)}$ cm^{-2} day^{-1}, respectively. The average water content of the cultivated BC pellicles in the unmodified and modified medium was about 98%–99%. The average

FIGURE 6.2 Bacterial cellulose–chitosan composite in the initial wet state of the never dried sheets.

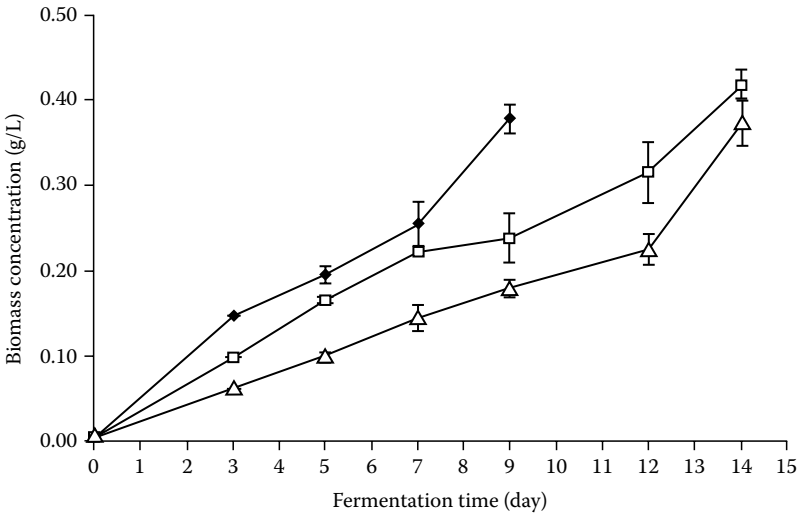

FIGURE 6.3 Growth of *Acetobacter xylinum* (AGR 60) in the coconut juice medium (◆) and coconut juice medium modified by chitosan MW 30,000 (□) and chitosan MW 80,000 (△).

cell growth rate of *A. xylinum* (AGR 60) in unmodified coconut juice medium was 0.042 $g_{(dry\ wt)}$ L^{-1} day^{-1}, which was comparable with the other strains cultivated in coconut juice medium (Kongruang 2008). However, with the supplement of chitosan MW 30,000 and 80,000 in the culture medium, the cell growth rates were significantly decreased to 0.030×10^{-3} and 0.027×10^{-3} $g_{(dry\ wt)}$ L^{-1} day^{-1}, respectively. Chitosan has been reported for the inhibition effects to cell growth of a wide variety of bacteria and fungi (Zhuang et al. 2008). The antibacterial effect of chitosan is dependent on the cationic nature, molecular size (Liu et al. 2006, Phisalaphong and Jatupaiboon 2008), the species of bacteria, concentration, pH, and so on (Huang et al. 2009). The supplement of chitosan MW 30,000–80,000 in the culture medium resulted in a significant decrease in cell growth rate of *A. xylinum* (AGR 60). Due to the slow cell growth rate, the cellulose formation in the modified culture medium with chitosan considerably decreased. Besides, the rate of BC formation is greatly dependent on bacterial strains, air–surface area, carbon and nitrogen sources, pH, and temperature. Table 6.1 presents the cell growth rates and the formation rates of cellulose and cellulose–chitosan composites in the coconut juice medium.

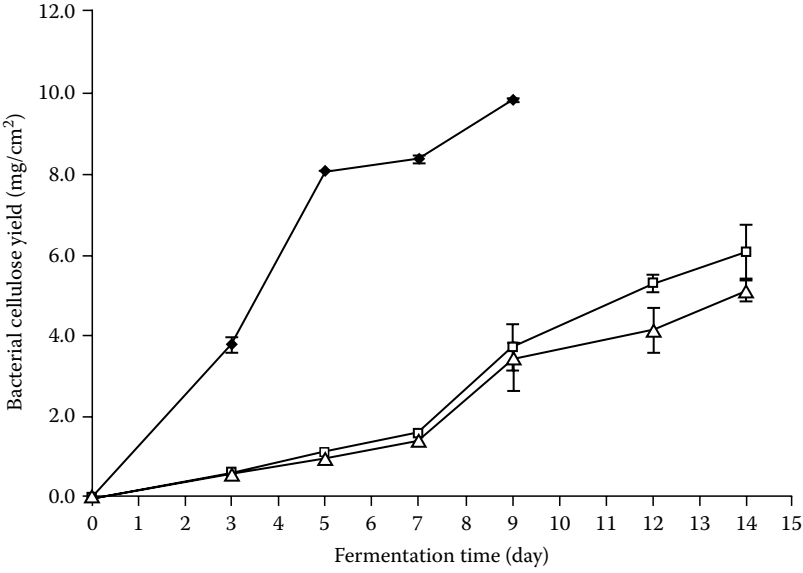

FIGURE 6.4 Bacterial cellulose yield (dry weight/interfacial surface area) by *Acetobacter xylinum* (AGR 60) in the coconut juice medium (◆) and coconut juice medium modified by chitosan MW 30,000 (□) and chitosan MW 80,000 (△).

TABLE 6.1
Rates of Cell Growth and Cellulose Formation of *A. xylinum* (AGR 60) in the Modified Coconut Juice Medium by Chitosan MW 30,000 and MW 80,000 in Comparison to Those in the Unmodified Coconut Juice Medium

Culture Medium	BC Production Rate ($g_{dry\ wt}$ cm^{-2} Day^{-1})	Cell Growth Rate (g L^{-1} Day^{-1})
Modified coconut juice medium by chitosan MW 30,000	0.43	0.030
Modified coconut juice medium by chitosan MW 80,000	0.37	0.027
Unmodified coconut juice medium	1.09	0.042

6.3 CHARACTERISTICS OF FILM

After biosynthesis, BC pellicles are usually purified by washing with DI water and then treatment with diluted NaOH solution to remove bacterial cells followed by a rinse with DI water. In wet state (never dried sheet), the SEM images of surface morphology of the modified BC pellicles by chitosan MW 30,000 and 80,000 are not significantly different from the unmodified BC as shown in Figure 6.5.

They all display ultrafine fiber network structure of microfibrils of below 0.01 μm. The average water content of the BC and the modified BC pellicles is 98%–99%. After the pellicles are air-dried at room temperature (30°C), the pellicles become transparent thin films. The SEM image of the air-dried films and the re-swollen films are shown in Figures 6.6 and 6.7, respectively.

With the modified chemical structure by chitosan, the network structures of both types of modified BC films become denser than those of the typical BC film and the pore sizes of films decrease

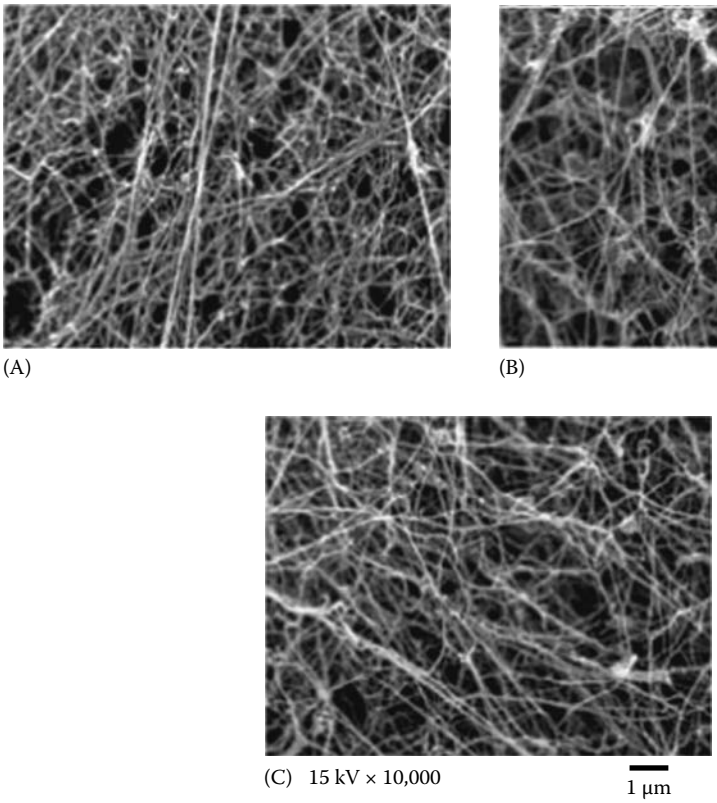

FIGURE 6.5 SEM images of cellulose network in the initial wet state of the never dried sheets of BC (A) and BC modified by chitosan MW 30,000 (B) and chitosan MW 80,000 (C).

with increasing the percent of chitosan (Phisalaphong and Jatupaiboon 2008). After water-reswelling, the apparent pore diameter of the swollen film is enlarged; however, the average pore sizes of the modified films in the re-swollen form are much smaller than those of the typical BC film.

BC is known for its remarkable capacity to hold water. The water content in the never dried BC sheets is about 95%–99%. The water absorption capacity of the BC film after air-drying at 30°C is about 480%–509% (Sanchavanakit et al. 2006, Phisalaphong and Jatupaiboon 2008), which is much higher than those of plant celluloses. It was found that with the incorporation of chitosan into the film, the water absorption capacity of the film was improved by about 1.4-folds (Phisalaphong and Jatupaiboon 2008). The water vapor transmission rate of the BC–chitosan films is 1560–1580 g m^{-2} day^{-1}, which is comparable to that of the typical BC. Its characteristics such as nanopore size, high surface area, high water absorption capacity, and maintaining moisture together with proper water vapor transmission rate are desirable properties for the application in wound dressing. Some physical characteristics of the BC and the BC–chitosan film are demonstrated in Table 6.2 (Phisalaphong and Jatupaiboon 2008, Kingkaew et al. 2010).

The modification of BC by chitosan was found to improve tensile strength and Young's modulus of the films in both dry and re-swollen forms; the increase of those mechanical properties was related to the content and molecular weight of chitosan (Phisalaphong and Jatupaiboon 2008). This result is rather similar to the composite cellulose membranes coated with chitosan (Yang et al. 2002). The fiber in the composite membrane would then withstand a stronger pull force than the cellulose fiber alone. However, the tensile strength in wet state is much lower than that in dry state due to the swelling of the cellulose–chitosan fibers in aqueous solution. Apparently, the modification by chitosan has a good

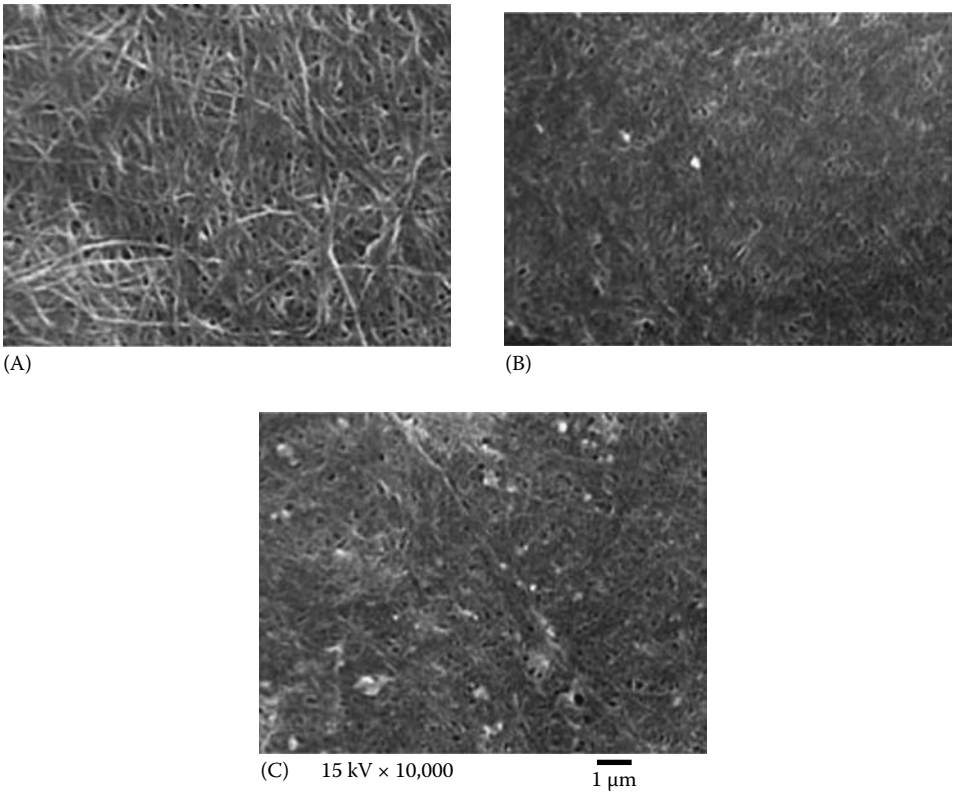

FIGURE 6.6 SEM images of surface morphology of the air-dried films of BC (A) and BC modified by chitosan MW 30,000 (B) and chitosan MW 80,000 (C).

impact on the mechanical properties of BC–chitosan films. Table 6.3 presents the mechanical properties of the BC and the modified BC films by chitosan and chitosan derivatives (Ciechańska 2004, Phisalaphong and Jatupaiboon 2008). The improved breaking stress of the modified BC by the supplement of chitosan acetate and chitosan lactate in culture medium was reported (Ciechańska 2004).

The toxicity and biocompatibility of the BC–chitosan composite were investigated (Kingkaew et al. 2010). The BC–chitosan film had no toxicity against L929 mouse fibroblast cell line and supported cell proliferation. Growths of human-transformed keratinocytes (HaCat) and human normal skin fibroblasts (CRL-2211) on the BC–chitosan film after 24 h of the cultivation in Dulbecco's Modified Eagle's medium were shown in Figures 6.8 and 6.9, respectively. The growths of human skin keratinocytes and fibroblasts on the BC–chitosan films were comparable to that on the BC film; however, the improvement of cell adhesion and spreading on the BC–chitosan films was observed in human skin keratinocytes (Kingkaew et al. 2010). The normal phenotypic shape suggested that the cells functioned biologically on these materials. Some physical and biological characteristics of two other types of hydrogels of BC–chitosan composites from microbial synthesis under static conditions in a standard Hestrin–Schramm culture medium supplemented with chitosan acetate and chitosan lactate were previously reported (Ciechańska 2004). The modified BC–chitosan composites presented a number of valuable characteristics such as improved mechanical properties, high moisture-keeping properties, release of mono-and oligosaccharides under lysozyme degradation and showed bacteriostatics activity against gram-negative and -positive bacteria. The BC–chitosan composites also showed some bactericidal activity against both *Escherichia coli* and *Staphylococcus aureus* (Ciechańska 2004). It was reported that BC modified with chitosan or chitosan derivatives combined properties of the two biopolymers such as bioactivity, biocompatibility, antimicrobial activities, and

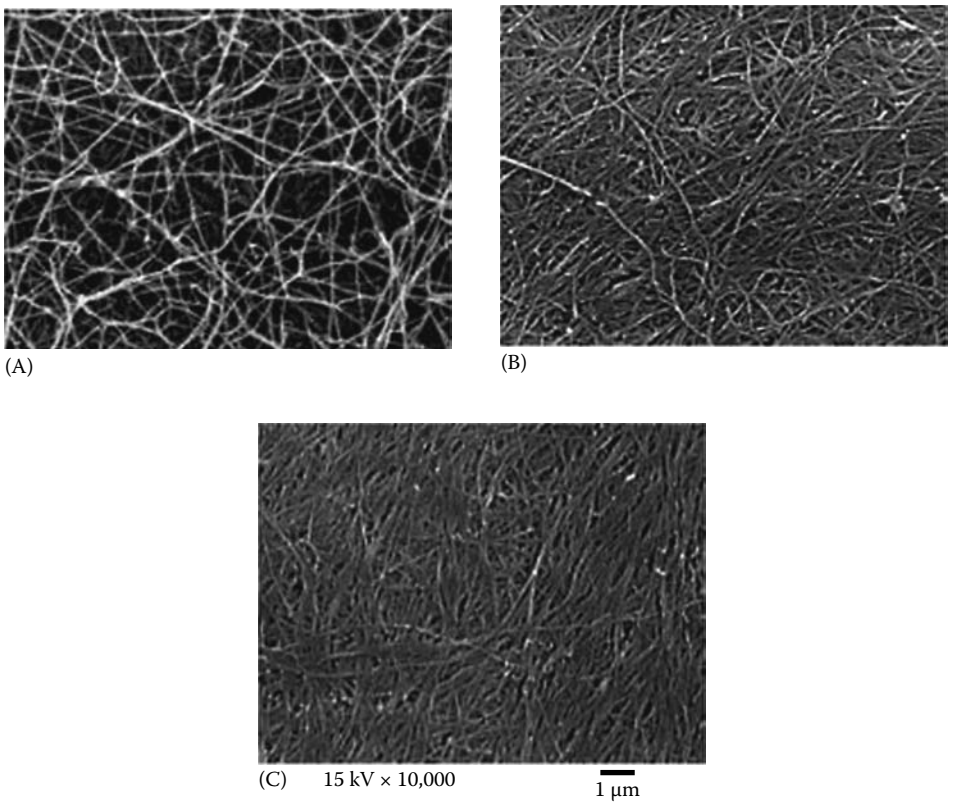

FIGURE 6.7 SEM images of surface morphology of the re-swollen films of BC (A) and BC modified by chitosan MW 30,000 (B) and chitosan MW 80,000 (C).

TABLE 6.2
Mechanical Properties of BC–Chitosan Films in Comparison to BC Films

Type of Films	State of Film	Tensile Strength (MPa)	Young's Modulus (MPa)	Elongation at Break (%)	Reference
BC	Wet	0.22		38	Ciechańska (2004)
BC/O		0.93		36	
BC/M		1.02		39	
BC	Dry	5.32	161.80	3.75	Phisalaphong et al. (2008)
BCC-3		7.66	195.00	1.91	
BCC-8		8.26	221.80	1.44	
BC	Re-swollen	1.45	21.79	8.17	
BCC-3		2.07	33.64	5.76	
BCC-8		2.72	36.13	4.36	

Note: BC, bacterial cellulose; BCC-3, BC modified by chitosan MW 30,000; BCC-8, BC modified by chitosan MW 80,000; BC/O, BC modified by chitosan acetate; BC/M, BC modified by chitosan lactate.

TABLE 6.3
Some Physical Properties of BC–Chitosan Films in Comparison to BC Film

	Dry Form			Re-Swollen Form		
	BC	**BCC-3**	**BCC-8**	**BC**	**BCC-3**	**BCC-8**
Pore diameter (nm)	22.4	15.1	13.2	61.2	48.6	40.1
Water absorption capacity (wt%)	482	606	652	—	—	—
Water vapor transmission ($g\ m^{-2}$ day)	1593	1578	1564	—	—	—

Note: BC, bacterial cellulose; BCC-3, BC modified by chitosan MW 30,000; and BCC-8, BC modified by chitosan MW 80,000.
The average thickness of films (in dry form) is 0.04 ± 0.005 mm.

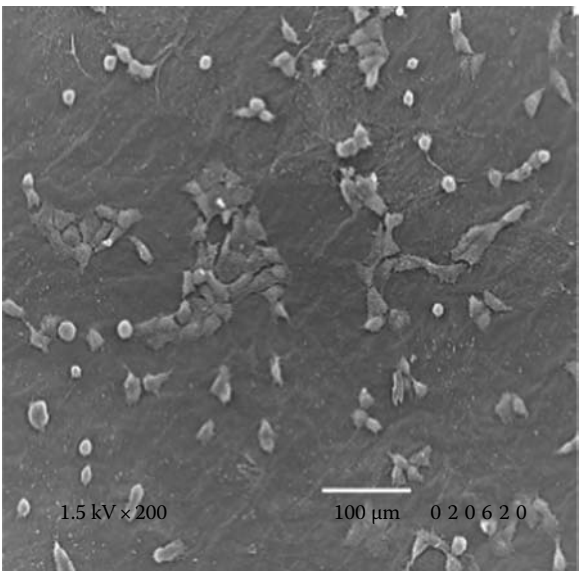

FIGURE 6.8 Growth of human-transformed keratinocytes (HaCat) on the bacterial cellulose–chitosan film demonstrated at 24 h.

biodegradability, which were key characteristics for wound dressing material (Ciechańska 2004, Petrulyte 2008, Fu et al. 2009, Kingkaew et al. 2010).

6.4 CONCLUSION

The modification of BC by means of adding different forms of chitin or chitosan in the culture medium during biosynthesis by *A. xylinum* produces valuable material. It is proposed that chitin or chitosan in the culture medium is depolymerized to monomeric and oligomeric during the incubation and is then utilized by *Acetobacter* as a carbon source. Glucosamine and *N*-acetylglucosamine units are incorporated into the polysaccharide chain by the metabolic pathway of glucose. The BC–chitosan composite combines the properties of both BC and chitosan. The film of BC–chitosan is homogeneous with a significantly denser fibril structure, smaller pore diameter, and higher surface area in comparison to those of typical BC films. The mechanical properties and the water

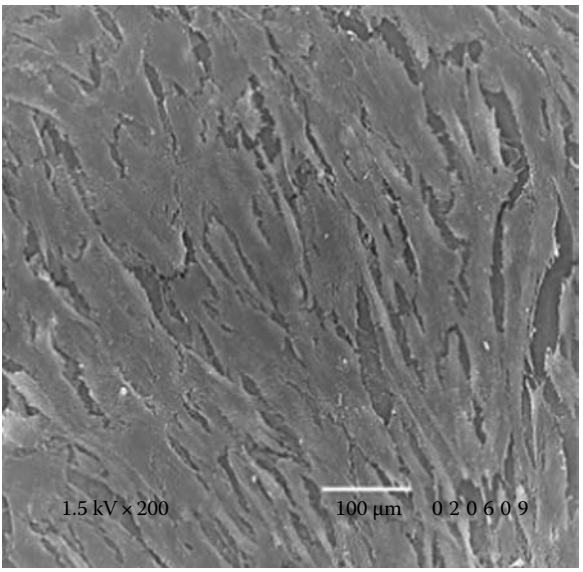

FIGURE 6.9 Growth of human normal skin fibroblasts (CRL-2211) on the bacterial cellulose–chitosan film demonstrated at 24 h.

absorption capacity of the modified films are significantly improved. Bioactivity tests reveal that the BC–chitosan composite has no toxicity and supports the growth, spreading, and adhesion of human keratinocytes and fibroblasts. These characteristics make BC–chitosan composite a good candidate for biomedical applications such as wound dressing material.

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Part II

Physical and Chemical Aspects of Chitin and Chitosan Derivatives

7 Chemical Derivatization of Chitosan for Plasmid DNA Delivery: Present and Future

Wing-Fu Lai and Marie Chia-Mi Lin

CONTENTS

7.1 INTRODUCTION

Chitosan is composed of randomly distributed glucosamine and *N*-acetyl-glucosamine monomers, and can be readily digested either by lysozymes or by chitinases (Aiba 1992, Hirano et al. 1988). Chitosan has been widely explored for its potential uses in a multitude of fields including cosmetics, food, sewage treatment, environmental protection, and biomedicine (Kim and Rajapakse 2005). Furthermore, because of its biodegradability, biocompatibility, nonallergenicity, and nucleic acid (NA)–binding ability, chitosan has recently been studied as a gene vehicle. To the best of our knowledge, the first team to explore the potential of chitosan for in vitro plasmid deoxyribonucleic acid (pDNA) delivery is Mumper et al. (1995), who revealed the theoretical possibility of chitosan in NA delivery and offered a foundation for later research.

In spite of the high potential of chitosan as a pDNA carrier, its poor solubility and low transfection efficiency have greatly impeded its practical application. Over the years, methods to boost the transfectability of chitosan have been studied a great deal and a more general understanding of parameters that may impact on the efficiency of transfection have been attained. However, the problem of comparatively low transfectability in chitosan vectors is yet to be utterly solved; consequently, more and more investigators have endeavored to chemically or biologically modify chitosan in order to overcome this Achilles' heel. In addition, though a vast variety of derivatization strategies have been explored to improve the water solubility (Kim and Rajapakse 2005) and transfection efficacy (Lai and Lin 2009) of chitosan, these efforts were scattered and diffuse, leaving viable directions for future research unclear.

In this chapter, we review the latest advances in chitosan modification, with a special focus on the synthesis and functional properties of chitosan/poly(ethylenimine) (CP/PEI) graft copolymers and their derivatives as pDNA carriers. We also address the prospects and challenges of the practical

application of chitosan-mediated pDNA delivery in the molecular sense. It is hoped that, with this chapter, a broader picture of the current developments of chitosan derivatization for pDNA transfer will be offered, and directions for future research would be implicated.

7.2 MAJOR CHEMICAL APPROACHES IN CHITOSAN DERIVATIZATION

Chemical derivatization in chitosan-based pDNA carriers was mainly performed based on the accumulated knowledge of factors influencing the transfectability of the polymeric pDNA carrier. Some of the commonly used strategies in chitosan derivatization include (1) functional group modification, (2) copolymerization, and (3) ligand conjugation. As the major advances in these strategies have been previously reviewed by Lai and Lin in 2009, in this and the following section we will give a brief overview of the much less discussed chitosan derivatives and put a special focus on the functional properties of CP graft copolymers and their derivatives as pDNA vehicles.

7.3 FUNCTIONAL GROUP MODIFICATION

The principle of functional group modification is to alter existing physical and chemical properties of chitosan in order to promote its capacity to deliver pDNA. Examples of vectors derived by functional group modification include thiolated chitosan (CSH) and *N*-acetylated chitosan.

Thiolated chitosan can be produced by a reaction between free amine groups of chitosan and carboxyl groups of thioglycolic acid, with the latter being activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. CSH displays better mucoadhesiveness and permeation enhancing effect in oral drug delivery (Bernkop-Schnurch et al. 2003, 2004, Roldo et al. 2004); however, studies on its potency as a gene carrier has long been lacking. Recently, Lee et al. (2007) evaluated the efficiency of CSH-mediated gene delivery, and noted that CSH with a higher thiol group content (360 μmol/g of chitosan) had better transfectability in HEK 293 cells. This may be linked to an increase in the number of attached thiol groups, which enhance cell permeation and hence the cellular uptake of the DNA–polymer complexes. At 60 h posttransfection, a drastic increase ($P < 0.01$) in transgene expression was observed in HEK 293 cells transfected with CSH 360 and cross-linked CSH 360 but not with Lipofectin or unmodified chitosan. It was therefore postulated that thiolation, with or without subsequent cross-linking, can reduce the positive charge density and pDNA complexing capacity of chitosan, thereby resulting in a more rapid release of pDNA from the complexes. However, the actual mechanism underlying the augmented transfection efficiency of CSH and its cross-linked derivatives has yet to be clarified. In in vivo studies, the level of cellular transfection in bronchoalveolar lavage (BAL) fluid was highest in CSH 360-mediated pDNA delivery 3 days after the intranasal administration, but in terms of sustained transgene expression, cross-linked CSH appeared to perform the best (Lee et al. 2007).

N-acetylated chitosan is another example of a functional-group-modification-derived chitosan-based pDNA carrier. Kai and Ochiya (2004) have previously reported its potential application in oral gene delivery. In their study, mice were fed with *N*-acetylated polyplexes carrying pDNA encoding *lacZ* and interluekin-10. The result of *lacZ* staining performed 5 days after transfection showed that transgene expression in duodenum, jejunum, ileum, and colon could only be mediated by *N*-acetylated chitosan but not standard chitosan. However, no evaluation on the toxicity of this chitosan derivative has yet been published. In order to further verify its workability in clinical use, studies on its toxicology and safety are in dire need.

7.4 COPOLYMERIZATION

Copolymerization is another common method for modifying or tailoring the physical, chemical, and/or biological properties of existing polymers for desirable functionality. An example of a derivative fabricated by this technique is stearic acid-graft-chitosan oligosaccharide (SA-g-COS), which,

in aqueous solution, was demonstrated to self-assemble micelles (Hu et al. 2006). In vitro studies showed that the transfectability of SA-g-CHI in A549 cells was sevenfold higher than that of chitosan, and was comparable to Lipofectamine 2000 (Hu et al. 2006). However, as the effectiveness of gene delivery mediated by SA-g-CHI has been assessed only in A549 cells, and the transfection efficacy of either Lipofectamine 2000 or SA-g-CHI in that cell line was relatively poor (20% and 15%, respectively), in order to further corroborate the potential of this derivative vector for pDNA transfer, in vitro studies in additional cell lines are needed.

Another example of chitosan-based pDNA carriers derived by copolymerization was chitosan–NIPAAm/vinyl laurate (VL) copolymer, which was reported by Sun et al. (2005), and was prepared by coupling a carboxyl-terminated *N*-isopropylacrylamide/VL copolymer with chitosan (Mw = 2 kDa). Despite its transfectability (at N/P ratios ranging from 2:1 to 20:1) being superior to naked DNA in C_2C_{12} mouse skeletal muscle cells, its maximal efficacy obtained was only 50% of that of Lipofectamine 2000. For the practical application of the vector in NA therapy, a further boost to its NA delivery efficiency is required.

7.5 LIGAND CONJUGATION

Ligand conjugation is the third most common strategy for chitosan derivatization. An example of a chitosan derivative developed by this approach is the chitosan–ethylene diamine tetraacetic acid (EDTA) conjugate. EDTA is a chelating agent commonly used to sequester di- and trivalent metal ions such as Mg^{2+}, Cu^{2+}, and Fe^{3+}. By virtue of its metal ion–chelating property, chitosan–EDTA conjugates are speculated to inhibit enzymes that required certain metal ion cofactors (such as DNAses that usually require Mg^{2+}), thereby protecting pDNA in the polyplex from nuclease degradation (Loretz and Bernkop-Schnurch 2006). With covalent attachment of EDTA to low-viscous chitosan (chitosan/EDTA weight ratio = 1:4, remaining chitosan NH_2 groups = 32%), Loretz and Bernkop–Schnurch recognized that the relative beta-galactosidase activity/mg lysate in Caco-2 cells transfected by chitosan–EDTA conjugates was approximately 7.5- and 1.5-fold that of standard chitosan and naked pDNA, respectively.

Apart from chitosan–EDTA conjugates, deoxycholic acid (DCA)–modified chitosan is derived by nonproteinaceous ligand conjugation. DCA is a secondary bile acid and a metabolic byproduct of intestinal bacteria. DCA is the most detergent-like physiological agent that can promote intestinal fat absorption by its emulsifying activity. Because of the hydrophobicity of DCA and the self-assembling properties of the bile acids in water, DCA was first used by Lee and Kim et al. for the hydrophobic modification of chitosan (Lee et al. 1998, Kim et al. 2001). Despite DCA-modified chitosan's ability to aggregate spontaneously to form micelles with an average diameter of around 160 nm, no significant augmentation on transfection potency could be observed in COS-1 monkey kidney cells after DCA incorporation.

Shortly thereafter, Chae et al.'s research group (2005) adopted the same strategy, but this time they used highly purified chitosan oligosaccharides (COSs) instead of standard chitosan as the backbone. They noticed that the conjugated polymer formed by COS (Mn = 3 kDa, 90.3% degree of deacetylation) with 25% DCA conjugation could yield pDNA expression in HEK 293 cells at least 20- and 100-fold higher than those transfected by poly-L-lysine (PLL) in the absence and presence of fetal bovine serum (FBS), respectively.

7.6 SYNTHESIS AND DERIVATIZATION OF CHITOSAN/POLY(ETHYLENIMINE) GRAFT COPOLYMERS

The CP copolymer, which was initially fabricated by cationic polymerization of aziridine in the presence of water-soluble oligochitosan (Wong et al. 2006), is one of the most widely studied derivative vectors in chitosan-mediated pDNA transfer. As the total number of hydrophilic amino groups on the chitosan backbone increases after PEI conjugation, the CP copolymer is generally more water

soluble than the parent chitosan. Luciferase activity assays revealed that the transfectability of the PEI-grafted chitosan was comparable to PEI 25 kDa both in vitro and in vivo (Wong et al. 2006).

Currently, Cho and his co-workers (Jiang et al. 2007a) synthesized a CP copolymer with its structure similar to Wong et al.'s derivative by an imine reaction between periodate-oxidized chitosan and PEI, and observed that, at high N/P ratios, the transfection ability of their copolymer could rival PEI 25 kDa and Lipofectamine in HeLa, 293T, and HepG2 cell transfection. In the presence of bafilomycin A1 (a specific inhibitor of vacuolar-type H^+-ATPase), their copolymer showed a drastic decrease in transfection efficiency in HeLa cells, but similar reduction was not observed when the transfection was mediated by chitosan. This implicates that the enhancement in in vitro pDNA transfer by chitosan after PEI conjugation came from the endo-lysosomalytic proton sponge effect of PEI.

PEI is one of the most extensively studied systems among different polymeric pDNA carriers. Chemically, PEI is a polymer, either in linear or branched forms, with one-third of the atoms being nitrogen and one-sixth of those nitrogen atoms being charged (Gao et al. 2007). Branched PEI can be synthesized by acid-catalyzed polymerization of aziridine either in alcoholic or aqueous solutions (von Harpe et al. 2000, Kunath et al. 2003), whereas linear PEI can be produced by complete hydrolysis of poly(2-ethyl-2-oxazoline) (Brissault et al. 2003). Compared to branched PEI, linear PEI was generally less cytotoxic, and more efficacious in transfection and nuclear localization (Zou et al. 2000).

With a literature search on PubMed and Medline, the number of papers on PEI was over 600, among which over 90% are working on the modification and derivatization of PEI for gene transfer. In fact, the research efforts paid by researchers in gene delivery technology was not demonstrated merely in the number of publications, but significant achievements in enhancement of polymeric vectors have also been obtained. For example, with PEGylation, the interparticular aggregation of PEI could be successfully inhibited, thereby alleviating the cytotoxicity of the polymer (Lee et al. 2001, Petersen et al. 2002). Apart from PEGylation, other modifications such as partial substitution of amino groups in PEI with imidazolyl moieties (Swami et al. 2007a) and cross-linking of PEI with bisepoxide (Swami et al. 2007b) were also shown to improve PEI's transfection efficiency and/or to reduce its cytotoxicity.

In order to determine the influence of the PEI moiety on the transfectability of CP copolymers, we attempted to conjugate PEI of different molecular weights (0.8, 1.3, 10, and 25 kDa) with conventional chitosan via the use of a coupling reagent, synthesizing copolymers CP0.8K, CP1.3K, CP10K, and CP25K, respectively. Based on the enhanced green fluorescence protein (EGFP) transfection assay on AGS cells in the presence of 10% FBS, the transfection efficiencies of our products were positively related to the molecular weight of the PEI grafted onto the chitosan backbone (Figure 7.1), and the transfectability of CP25K was superior to CP10K, CP1.3K, and CP0.8K. This might be explained by an increase in the proton sponge properties of the CP copolymer when the molecular weight of its PEI moiety is increased. On the other hand, the higher the molecular weight of the PEI moiety, the more cytotoxic the synthesized CP copolymer. Because of this, based on the degree of morphological changes experienced by the transfected cells as viewed under the microscope, CP25K was recognized to be by far the most toxic candidate among a panel of products being examined in vitro, followed by CP10K, CP1.3K, and CP0.8K. Based on this evidence, we could see that the molecular weight of the PEI moiety adopted could possibly be one of the pivotal factors determining the transfectability of the CP copolymer produced. A trade-off between the cytotoxicity of the copolymer and its transfection efficiency might be of practical importance in optimization of the fabricated CP copolymer.

In addition to the effect of the PEI moiety, we have also compared the effect of two chemical modification methods (*N*-methylene phosphonation and quaternization) on the transfectability of the CP copolymer. *N*-methylene phosphonation is a chemical approach to increase the water solubility of chitosan via the introduction of the phosphonic acid function (Heras et al. 2001); whereas quaternization is an old process that comprises methyl iodide–mediated *N*, *N*, *N*-trimethylation for the fabrication of chitosan soluble in water at the physiological pH (Thanou et al. 2002). In weighing

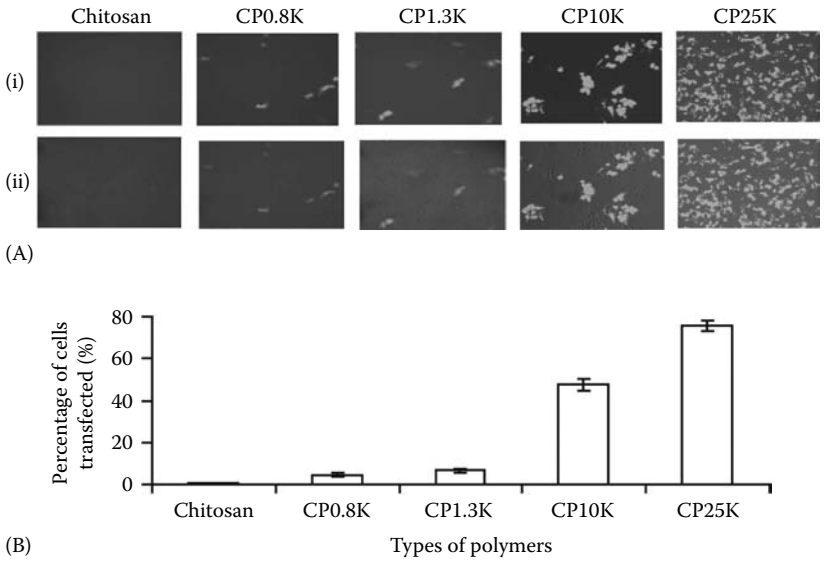

FIGURE 7.1 (A) Representative (i) EGFP fluorescence images and (ii) phase-contrast images of AGS cells at 48 h after transfection mediated by a panel of chitosan-based polymers (chitosan, CP0.8K, CP1.3K, CP10K, and CP25K) at their corresponding optimal polymer/DNA mass to mass ratios in the presence of 10% FBS. (B) The percentage of the transfected AGS cells expressing the EGFP protein as estimated by averaging the cell counts of five randomly chosen views under the fluorescence microscope.

the cytotoxicity and transfection efficiency of different CP copolymers examined in Figure 7.1, we chose CP10K to be the starting material for further derivatization. The products derivatized by *N*-methylene phosphonation and quaternization are designated as PCP-10K and QCP-10K, respectively, and their structures are shown in Figure 7.2. Based on our result (Figure 7.3), it appeared that no significant difference on the transfectability between unmodified CP10K and the derivatized products (PCP-10K and QCP-10K) was observed. This is possibly because chitosan's proton-sponge properties, water solubility, and hence the overall transfection efficiency have already been largely improved during PEI conjugation when the CP copolymer is fabricated, further improving the water solubility of the chitosan backbone in the copolymer via these two derivation strategies may not significantly boost the transfectability of the product any further. In fact, due to the potential changes in the biological properties of CP10K after functional group modification, the cytotoxicity of the copolymer was enhanced after derivatization. This was manifested by the degree of morphological changes exhibited by the transfected cells as viewed under the microscope.

In fact, *N*-methylene phosphonation and quaternization are only some examples of derivatization approaches for chitosan. As light has already been shed on the feasibility of adopting chitosan and PEI together as a combination in copolymerization for pDNA transfer, more and more efforts have now be paid on further derivatization of CP copolymers for performance and functionality optimization. For instance, in 2002, Li et al. have galactosylated the CP copolymer to enhance its target-specificity to galactose-recognizing asialoglycoprotein receptors, and have hence improved the efficiency of the product in HepG2 transfection (Jiang et al. 2007b).

Folic acid conjugation is also a derivatization method potentially applicable to CP copolymer modification for target specialty toward cancerous cells. The underlying rationale pertains to the relatively strong binding of folic acid to folate receptors (FRs) overexpressed on carcinoma. In fact, in virtually all bodily tissues, the reduced folate carrier (RFC) is expressed. RFC is presumed to be the major route for the uptake of reduced folate cofactors such as 5-methyltetrahydrofolate (5MTHF), which accounts for approximately 99% of folates in our circulation. In addition to RFC, another type of folate transporters for cellular folate uptake is FRs, which can principally be

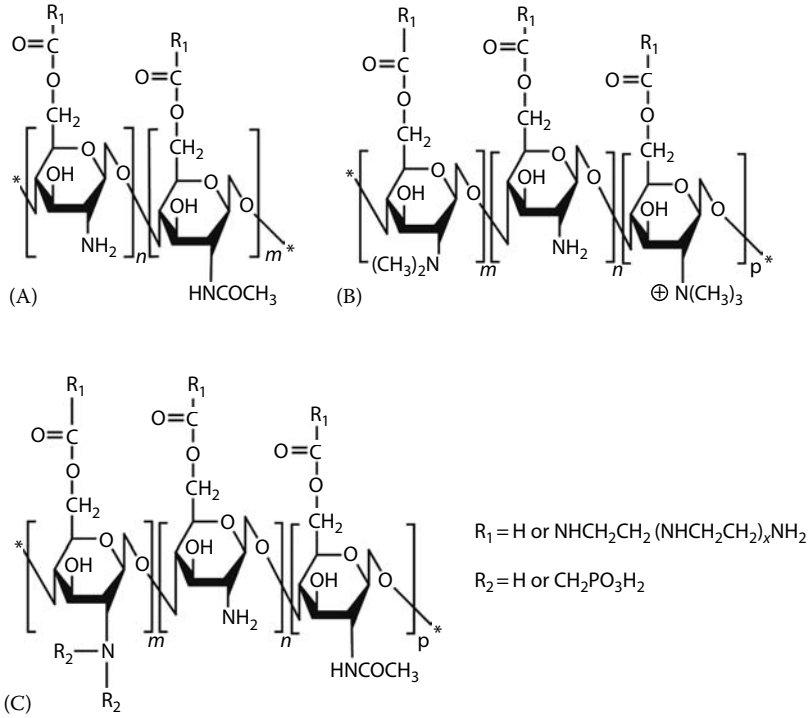

FIGURE 7.2 The structures of (A) CP10K, (B) QCP10K, and (C) PCP10K.

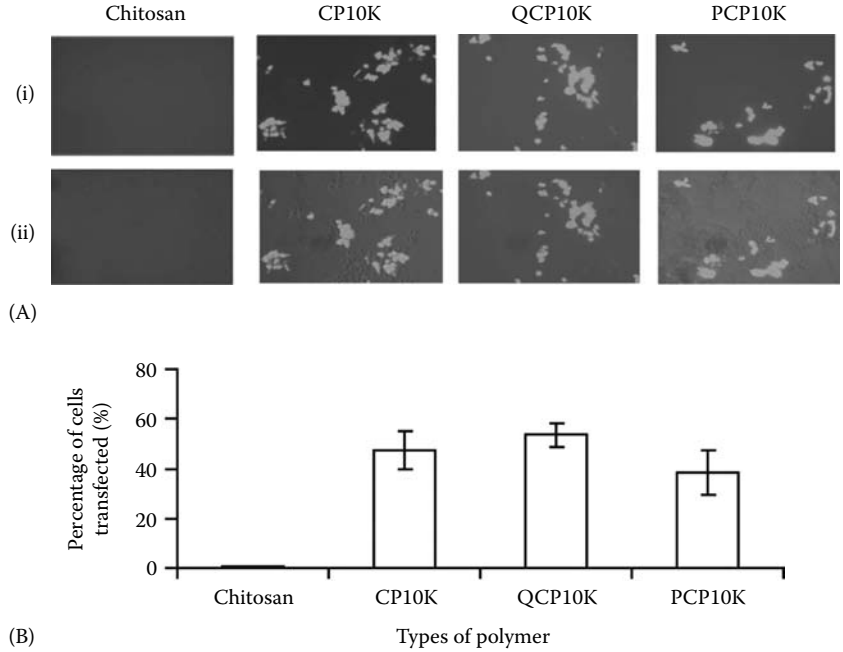

FIGURE 7.3 (A) Representative (i) EGFP fluorescence images and (ii) phase-contrast images of AGS cells at 48 h after transfection mediated by a panel of chitosan-based polymers (chitosan, CP10K, QCP10K, and PCP10K) at their corresponding optimal polymer/DNA mass to mass ratios in the presence of 10% FBS. (B) The percentage of the transfected AGS cells expressing the EGFP protein as estimated by averaging the cell counts of five randomly chosen views under the fluorescence microscope.

subdivided into three isoforms (namely FRα, FRβ, and FRγ) (Matherly and Goldman 2003), and among them, FRα has gained the most attention because it is primarily expressed in the cells of tumors such as malignant nasopharyngeal, colon, ovarian, breast, renal, and testicular carcinomas (Elnakat and Ratnam 2004, Theti and Jackman 2004). In contrast to RFC, FRα has much lower affinity to 5MTHF, but binds to folic acid strongly. As FA is a high-affinity ligand of FRα and can possibly retain its receptor-binding affinity upon derivatization, FA has been conjugated to miscellaneous polymeric pDNA carriers to promote the uptake of polyplexes by cancerous cells (Shi et al. 2002, Hattori and Maitani 2005, Chan et al. 2007).

In 2006, Mansouri and his coworkers fabricated FA-chitosan-DNA (FA-CHI-DNA) nanoparticles by reductive amidation and complex coacervation. By the 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay, they found that in HEK 293 cells, chitosan–DNA nanoparticles with or without FA conjugation (w/w = 1/20) exhibited similar cytotoxicity (~80% cell viability 60 h posttransfection), whereas in the case of Lipofectamine 2000, only 30% cell viability was attained. Shortly thereafter, Chan et al. (2007) attempted to conjugate folate to poly(ethylene glycol)-grafted chitosan (FA-PEG-CHI). The product with the degree of substitution (d.s.) above 6% was shown to be highly soluble in physiological pH. In addition, the low cytotoxicity of FA-PEG-CHI/DNA complexes (N/P = 20, cell viability >90% after 72 h of incubation) in HEK 293 was comparable to Mansouri et al's FA-CHI-DNA nanoparticles. However, no solid in vitro data on FA-PEG-CHI/DNA complexes and FA-CHI-DNA nanoparticles have yet been published by either research team. The efficiency of these two vectors in pDNA transfer is still a myth regardless of their potential as pDNA carriers as demonstrated by their DNA condensing abilities.

Recently, Jiang et al. (2009) have attempted to conjugate folic acid onto CP copolymers by first conjugating FA to chitosan via 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC)–mediated coupling reaction, followed by an imine reaction between periodate-oxidized folate–chitosan (FC) and PEI (Mw = 1.8 kDa). In a urethane-induced lung cancer mouse model, they demonstrated that aerosol delivery of Akt1 shRNA mediated by the copolymer as an RNA carrier could suppress lung tumorigenesis through the Akt signaling pathway. Although data on its efficacy for pDNA delivery has yet to be collected, its in vivo transfectability on mice has already shed light on its potential for NA therapy.

7.7 PROSPECTS AND CHALLENGES

The potential of chitosan in therapeutic small RNA transfer and oral/nasal NA therapy have been previously discussed by Lai and Lin in 2009. In this section, we are going to explore additional prospects of chitosan-mediated pDNA delivery in the molecular sense: corrections of genomic mutations and the rejuvenation of the degenerated nervous system.

The accumulation of mutated genes is a cellular problem that can ultimately lead to carcinogenesis and/or cellular malfunctioning. In order to tackle the problem, one can either knockdown the mutated gene or introduce a transgene that can induce apoptosis in mutated cells, thereby preventing any subsequent unwanted cellular events such as tumorigenesis. To accomplish this kind of genetic manipulation, NA therapy is possibly a way out. Apart from its therapeutic potential toward cancer and cellular physiological disorders, the prospect of genetic manipulation via NA therapy for the rejuvenation of the degenerated nervous system also seems to be worth paying attention to. In fact, the association between aging and its neuro-anatomical changes both in humans and laboratory animals has been extensively studied since 1900s, from which observations have been made on neuron loss in the cerebral cortex (Tomlinson 1976) and cerebellar cortex (Hall et al. 1975), and the slowing down of motor nerve conductance with advanced age (Kemble 1967). Moreover, aging has been found to be associated with the progressive increase in the incidence of a number of neurodegenerative disorders including Parkinson's disease and Alzheimer's disease, which are related to the degeneration of nigrostriatal dopaminergic neurons and the atrophy of cholinergic neurons, respectively. In view of the age-associated physiological

changes in the nervous system, it is believed that in order to rejuvenate the functions of the aging nervous system, two levels of actions might be required.

The first level is to prevent aging-associated neurodegeneration via neuroprotection, and this could possibly be attained by genetic manipulation of neuronal cells. This is suggested experimentally by the fact that mice with experimental autoimmune encephalomyelitis could have less retinal ganglion cell loss, optic nerve demyelination, axonal loss, and cellular infiltration six months after intraocular injections of adeno-associated viral (AAV) vector expressing the human extracellular superoxide dismutase (ECSOD) or catalase (CAT) gene (Qi et al. 2007). Such plausibility of artificial gene expression modification for neuroprotection was further supported by the fact that the overexpression of certain proteins in the central nervous system may protect neurons from death and spare neurological functions after neurological insult. This can be exemplified by the observation that the overexpression of either the Glut-1 glucose transporter or the apoptosis inhibitor Bcl-2 can decrease hippocampal neuron loss during excitotoxic insults (McLaughlin et al. 2000). Barring the Glut-1 and Bcl-2 glucose transporter, proteins like GDNF (Costantini et al. 1999, Simonato et al. 2000), tyrosine hydroxylase (Costantini et al. 1999, Simonato et al. 2000), calcium-binding proteins (Kindy et al. 1996, Meier et al. 1997), anti-apoptotic proteins (Costantini et al. 1999, Simonato et al. 2000), anti-oxidant enzymes (Kindy et al. 1996), and anti-inflammatory proteins (Betz et al. 1995) have also been shown to exhibit different levels of neuroprotective effects when they are overexpressed. These observations verify the feasibility of genetic manipulation for neuroprotection.

The second level of interventions for nervous system rejuvenation is neurological regeneration. There are a number of factors possibly pertaining to diminished axonal regenerations and/or synaptic decline. These include gene silencing (Burzynski 2003), chromosomal changes (Ikura and Ogryzko 2003), decrease in RNA polymerase levels (da Silva et al. 2000), intracellular deposition of metabolic wastes (Bendiske et al. 2002), microtubule destabilization (Bendiske et al. 2002), and a lack of trophic factors (Chao 2003). As some of the above factors are caused by aging-related gene expression level changes in neuronal cells, one of the potential strategies to achieve neurological regeneration is the replacement of inactivated genes. The rationale of stimulating the regeneration of nerves or nonactive tissues (such as bone) by the modulation of the gene expression profile was supported by nerve regeneration in neurotomized mice after receiving a local injection of the vascular endothelial growth factor (VEGF) gene (Fu et al. 2007). In addition, the viability of artificial axonal regeneration were also exhibited in bone marrow stromal cells (Koda et al. 2007) and in spinal cord–transected rats (Koda et al. 2004) after receiving the adenoviral delivery of the brain-derived neurotrophic factor (BDNF) gene. These experimental observations not only revolutionalize our conventional understanding of neurophysiology but also provide us insights on the restoration of damaged neuronal tissues via genetic manipulation.

Judging from the previously discussed scientific evidence, we have confidence to believe that corrections of genomic mutations and the rejuvenation of the nervous system could at least be theoretically possible if manipulation at the genetic level can be achieved; however, it is worth noting that in order to put such a theoretical concept into practice, a number of challenges have to be resolved beforehand. An example of these challenges is a lack of efficient gene carriers for the introduction of exogenous genes and hence cellular genetic manipulation. Over the past 30 years, copious polymeric gene carriers have been studied (Boussif et al. 1995, Chemin et al. 1998, Goula et al. 1998, Davis 2002, Sun et al. 2003, Pearson et al. 2004, Je et al. 2006), but the search for clinically applicable vectors for human use is still the biggest hurdle in pDNA delivery. Similar to other polymeric vectors, one of the difficulties in efficient chitosan-mediated pDNA delivery is the presence of different cellular barriers (including enzymatic degradation, inefficient cellular uptake, encapsulation in endo-lysosomes, failure of pDNA/polymer dissociation, and nuclear localization) during the course of pDNA transfer (Koping-Hoggard et al. 2001, Davis 2002, Kaneda 2005). The simplified mechanism of polymeric pDNA delivery and some cellular barriers involved are presented in Figure 7.4.

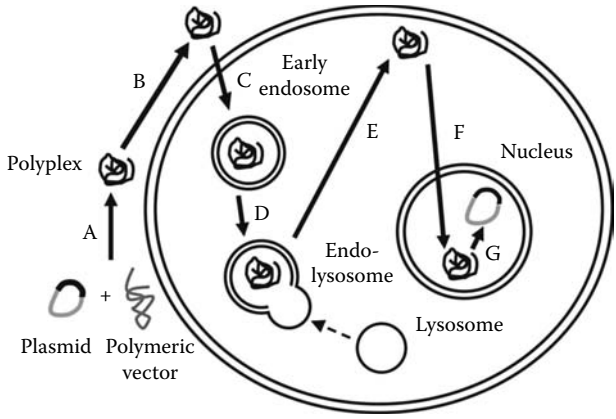

FIGURE 7.4 The simplified mechanism of polymeric NA delivery: formation of polyplexes (A); internalization contact (B); cellular uptake (C); encapsulation in the endosome (D); endo-lysosomal escape (E); nuclear localization (F); dissociation of DNA from the vector (G).

Further, at present the design of pDNA delivery vectors is largely trial and error by nature (Lai and Lin 2009), and such an approach is slow and inefficient. In order to resolve the situation, an organized approach in vector development appears to be a sine qua non, and this might possibly be achieved via QSAR (qualitative structure–activity relationships). In QSAR, the relationship between pharmacological activities of a compound and its chemical structure are described and predicted based on some well-established statistical tools and descriptors (e.g., shadow indices and molar refractivity, molecular surface area, encode molecular connectivity). With the use of QSAR in vector design, this not only can shorten the time to screen out and optimize candidates in experiments but also can largely rationalize the vector optimization process, thereby facilitating the progress of vector development. For more detailed discussions about the prospect of QSAR in vector design, please refer to the review offered by Lai and Lin (2009).

In addition to a lack of an efficient pDNA carrier, another factor posing challenges and complexity to the success of genetic manipulation is the subtle interaction between genes and the milieu in which genes operate (Rattan 2007). In fact, both "stochastic epigenetics" and "regulated epigenetics" have played significant roles in the onset of gene actions. Some of the factors involved in epigenetic modulation include hormonal and nutritional status, prenatal maternal health, and postnatal exposure to bacteria, viruses, and other germs (Mueller et al. 2007, Parsons 2007, Hipkiss 2008, Yilmaz et al. 2008). In view of such a complexity, despite the sophistication of genetic manipulation being important for causing alternations at the molecular level, the ability of properly controlling the milieu in which genes operate would be the actual factor that eventually determines the success or failure of the technology.

Furthermore, most of the physiological changes (like cancers aging and the degeneration of nerves) caused by genetic changes are polygenic traits. In order to correct those changes at the molecular level, we first have to understand how many genes or their variants would have to be manipulated. Unfortunately, at present there is a vast knowledge gap on the interactions of different genes and on the long-term physiological price paid by the subject when the action of each gene is modified. The presence of such a gap is partially resulted from the fact that most of the studies in the field so far have mainly focused on individual genes or proteins instead of looking at the actual interactions between them. Therefore, for future research, a more comprehensive approach to attain a fuller picture of gene interactions should be emphasized. In addition to the aforementioned difficulties, there may also be challenges specific to individual genetic interventions (e.g., a lack of efficient NA vehicles to pass through the blood–brain barrier for applications in neurological rejuvenation). All of these problems are worth additional research efforts for advancing the field to the next level.

Solving all the problems mentioned above would not be a light task; however, new light has already been shed on genomic and/or phenotypic manipulation in vitro via pDNA delivery mediated by chitosan and other polymers. Recently, with the use of chitosan-mediated oral NA transfer, Bowman et al. (2008) successfully attained phenotypic correction in over 50% of hemophilia A mice after the oral administration of FVIII-encoding pDNA encapsulated in chitosan nanoparticles. According to their results, the expression of the transgene was found in both local gastrointestinal tissues (such as stomach, ileum, and Peyer's patch) and systemic tissues (including liver and spleen). As demonstrated by chromogenic and thrombin generation assays, a peak level of 2%–4% functional factor VIII protein was obtained in plasma 22 days after in vivo administration (Bowman et al. 2008). Although the copy number of the plasmid per cell achieved in organs such as the liver, stomach, ileum, and spleen was still low (0.02 or below) in their study, the theoretical plausibility of genetic attenuation have already been confirmed. What remains is the improvement of existing technologies and scientific tools to accomplish the goal.

In addition to chemical means, pDNA delivery via physical means such as electroporation has also gained advancements. For instance, by changing the pulsed electrical fields and subsequent pressure from caliper-type electrodes on topically applied naked *lacZ* gene constructs, electrically controlled depth-targeted delivery of the *lacZ* reporter gene was successfully achieved in different dermis regions in hairless mice (Zhang et al. 1996). Recently, a group has also reported that the administration of IL-12 encoding pDNA via intramuscular electroporation could inhibit the growth of tumors with a diameter of less than 3–5 mm, and hence extend the survival rate of the mice models (Zhu and Li 2008). Although more studies would be required in order to allow genomic manipulation to be widely used in clinical sectors, the success of these studies have already revealed the possibility of treating diseases or even aging-associated symptoms (such as wrinkling of the skin or changes in the expression of some vital genes) at the molecular level. Once an efficient chitosan-mediated pDNA carrier or other pDNA delivery approaches are developed, it is conceivable that vistas of further advances in clinical medicine would be more easily achieved due to the possibility to manipulate the functioning of the human genome.

7.8 CONCLUDING REMARKS

Chitosan has been widely explored for possible uses in a variety of areas including pDNA delivery. Although the search for an efficient chitosan vector might still be an uphill struggle over the forthcoming decade, with the accumulated efforts of previous research, light has already been shed on how the transfectability of chitosan vectors could possibly be augmented. It is believed that such general understanding of chitosan could potentially be a source of knowledge for researchers in vector design, thereby promoting the development of technologies in chitosan-mediated DNA transfer in the future.

In this chapter, we have highlighted some of the prospects of chitosan-mediated pDNA delivery technologies in corrections of genomic mutations and the rejuvenation of the degenerated nervous system as potential directions of future research; however, relevant sections of this chapter are only intended to be the lead-in to those topics. We welcome other researchers to share their experiences and insights concerning those fields. We believe that through collaborative research efforts made by scholars with a variety of expertise, significant advances in chitosan-mediated pDNA delivery techniques could be made in the foreseeable future.

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8 X-Ray Diffraction Studies of Chitin, Chitosan, and Their Derivatives

Waldemar Maniukiewicz

CONTENTS

8.1 INTRODUCTION

Scattering is a powerful analytical method that can be used to determine the structure and dynamics of a range of "soft" materials like colloids, polymers, proteins, etc., using x-rays, neutrons, electrons, or visible light. X-ray crystallography (also called x-ray diffraction) is the most straightforward method of determining the arrangement of atoms within a crystal, in which a beam of x-rays hits a single crystal and diffracts into many specific directions. From Bragg's angles and intensities of these diffracted beams, one can produce a three-dimensional picture of the density of electrons within the crystal. Then, from the electron density, the mean positions of the atoms in the crystal can be determined, as well as the various other structural information. In the case of polysaccharides such as chitin or chitosan, due to anisotropic growth rate, sufficient single crystals for diffraction experiment cannot be obtained easily. In addition, the crystallinity of studied "soft" material is sometimes only partial. This means that there are severe dislocations in the crystal lattice or some amounts of amorphous material, which make the interpretation of the diffraction pattern obscure. Fortunately, polysaccharides often form polycrystalline and/or orientated fibers in which the axes of the long polymeric structures are parallel to each other. So, various other x-ray diffraction techniques can be applied to obtain valuable information about the structure; such methods include fiber x-ray diffraction, powder diffraction (WAXS), and small-angle x-ray scattering (SAXS). With data emerging from not less than 20 books, over 300 reviews, over 12,000 articles, and innumerable patents (Pillai et al. 2009), the aim of the present review is to present a state-of-the-art study on crystal structure of chitin, chitosan, and their derivatives.

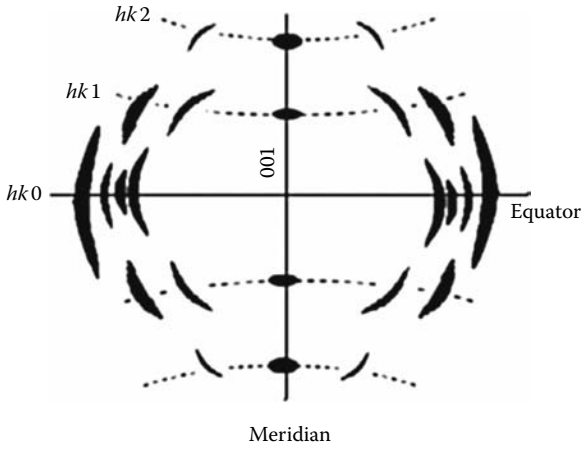

FIGURE 8.1 Idealized fiber diffraction pattern of a semicrystalline material with reflexions on layer lines. High intensity is represented by dark color. The fiber axis is vertical.

8.1.1 Fiber X-Ray Diffraction

A fiber x-ray diffraction experimental setup is rather simple: the orientated fiber is placed in a collimated x-ray beam at right angles to the beam and the diffraction pattern (Figure 8.1) is recorded on a film or detector (i.e., imaging plate) placed a few centimeters away from the fiber. Since fibers show helical symmetry rather than the three-dimensional symmetry shown by crystals, the strategy to determine polysaccharide crystal structure usually involves multiple stages. First, the most important parameters such as those defining the chain packing positions are searched. Then, less important parameters are gradually introduced as the structure refinement proceeds (Dumitriu 2005). Otherwise, it is very likely that the structure search can be trapped in local minima owing to the poor quality of fiber diffraction data compared with data derived from single crystals. Furthermore, to complement diffraction data of low resolution, proposed crystal structure may be evaluated in terms of not only diffraction data but also stereochemical criteria. Structure determination and refinement is usually carried out using a computer program, that is, the linked-atom least-squares program (Campbell and Arnott 1978).

8.1.2 X-Ray Powder Diffraction

The powder diffraction experiment (WAXS) remains a crucial tool in the characterization of materials, and it has been used for many decades with increasing importance and breadth of application as instrumentation, methods, data analysis, and modeling become more powerful and quantitative. Although powder data usually lack the 3-D of the diffraction image, the fundamental nature of the method is easily appreciated from the fact that each powder diffraction pattern represents 1-D snapshot of the 3-D reciprocal lattice of a crystal. The quality of the data is usually limited by the resolution of the powder diffractometer and by the physical and chemical conditions of the specimen (Figure 8.2).

8.2 CHITIN STRUCTURE IN THE SOLID STATE

8.2.1 General Remarks

Chitin, a linear polysaccharide of β-(1→4)-2-deoxy-2-acetamido-D-glucopyranose, is a major polysaccharide found in crustaceous shells and in cell walls of fungi. In its chemical structure, chitin is similar to cellulose, but it is different in that it has an acetamide group instead of a hydroxyl group

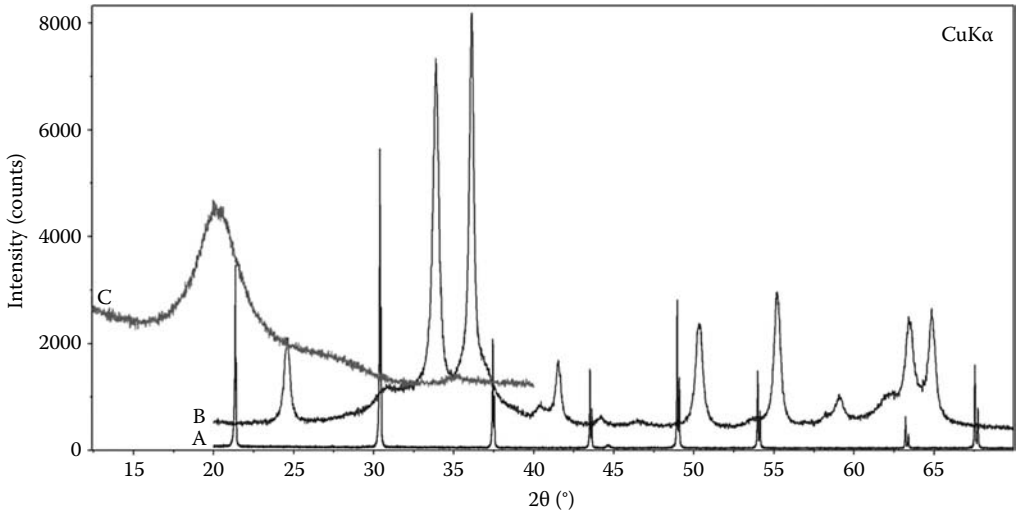

FIGURE 8.2 Powder diffraction patterns collected from three different samples. Pattern A represents a material with excellent crystallinity. Pattern B is also crystalline material; however its crystallinity is poor. Pattern C is collected from noncrystalline material.

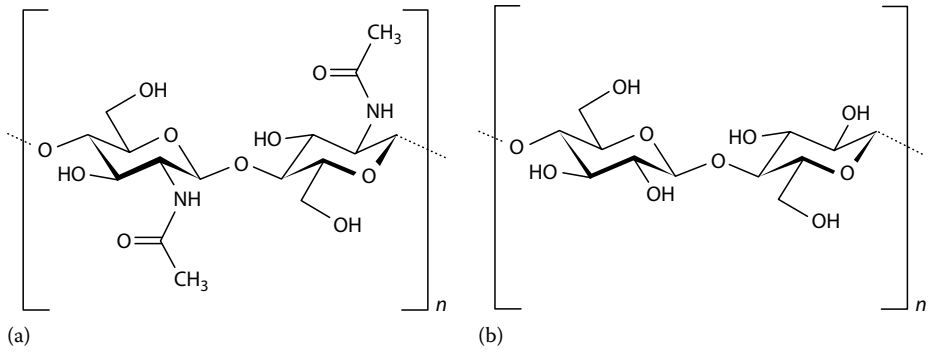

FIGURE 8.3 Chemical structure of (a) chitin and (b) cellulose.

at the C-2 position within the glucose unit (Figure 8.3). Samples of the native chitin may contain broadly different amounts of *N*-acetyl groups, depending on their origin and isolation procedure. In general, the degree of *N*-acetylation of native chitin is in the range 0.90–0.95. So far, it has been reported that chitin, depending on its source has three anhydrous crystalline polymorphs, α-, β-, and χ-chitins, in the native state (Rudall 1963, Gardner and Blackwell 1975, Minke and Blackwell 1978). The β- and χ-chitin forms are irreversibly converted into the α-form, which is the most abundant of the three polymorphs.

The α- and β-forms of chitin can be considered as analogues of cellulose II and I, respectively, in terms of chain packing, and their structural details they have been well characterized (Rao et al. 1998). The α-chitin posses a tightly compacted orthorhombic cell formed by alternated sheets of parallel and antiparallel chains (Minke and Blackwell 1978, Jang et al. 2004). The β-chitin adopts a monoclinic unit cell where the polysaccharide chains are disposed in a parallel fashion (Gardner and Blackwell 1975), and although the structure of χ-chitin have not been completely identified, an arrangement of two parallel chains in association with one antiparallel chain has been proposed (Rudall 1967, Gardner and Blackwell 1975, see Figure 8.4. Though, it has been also suggested that χ-chitin may be a distorted version of either α- or β-chitin rather than a true third polymorphic form (Roberts 1998).

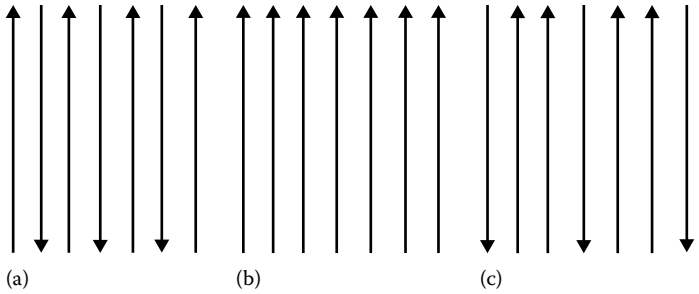

FIGURE 8.4 Three polymorphic configurations of chitin: (a) α-chitin, (b) β-chitin, and (c) χ-chitin.

TABLE 8.1
Crystallographic Parameters of α- and β-Chitin

	a (Å)	*b* (Å)	*c* (Å)	χ (°)	Space Group	Reference
α-Chitin	4.74	18.86	10.32	90	$P2_12_12_1$	Minke and Blackwell (1978)
	4.71	18.78	10.30	90	$P2_12_12_1$	Sikorski et al. (2009)[a]
β-Chitin	4.85	9.26	10.38	97,5	$P2_1$	Gardner and Blackwell (1975)

[a] Data collected at 100 K.

The currently accepted unit cell parameters and symmetry for α-chitin and β-chitin are listed in Table 8.1.

According to Rudall (Rudall 1967), α-chitin has strong intersheet and intrasheet hydrogen bonding, and β-chitin has weak hydrogen bonding by intrasheets. Therefore, in contrast to α-chitin, β-chitin is characterized by a weak intermolecular force (Rudall and Kenchington 1973), and it has been confirmed to exhibit higher reactivity in various modification reactions and higher affinity for solvents than that of α-chitin (Kurita et al. 1993, 1994).

8.2.2 Crystal Structure of α-Chitin

The crystal structure of α-chitin has been the subject of many crystallographic investigations, dating from the middle of 1930s. Early work established the unit cell parameters and probable space group (Mayer and Pankow 1935, Clark and Smith 1936). In 1957, Carlström (Carlström 1957) proposed the structure of α-chitin from a combination of x-ray and optical studies. The currently most accepted crystal structure of α-chitin has been proposed by Minke and Blackwell (1978). Very recently, Sikorski et al. (Sikorski et al. 2009) redetermined the crystal structure of α-chitin using high-resolution synchrotron x-ray fiber diffraction data. Experimental data were collected at 100 and 300 K. The main features of the crystal structure are in general agreement with those proposed by Minke and Blackwell. The unit cell contains two polymer chains in a 2_1 helix conformation and in the antiparallel orientation. The asymmetric unit consists of only a single pyranose unit. The polymer chains form hydrogen-bonded sheets linked by C7=O7·····H–N hydrogen bonds parallel to the *a*-axis (Figure 8.5). In addition to O3–H·····O5 intramolecular hydrogen bond, the CH_2OH side chain forms several hydrogen bonds to the carbonyl oxygen on the next chain. These results also acknowledged that a statistical disorder of hydroxymethyl group is present in the crystal. The two distinctive conformations of C6–O6 are, however, different from what has been suggested by Minke and Blackwell (1978).

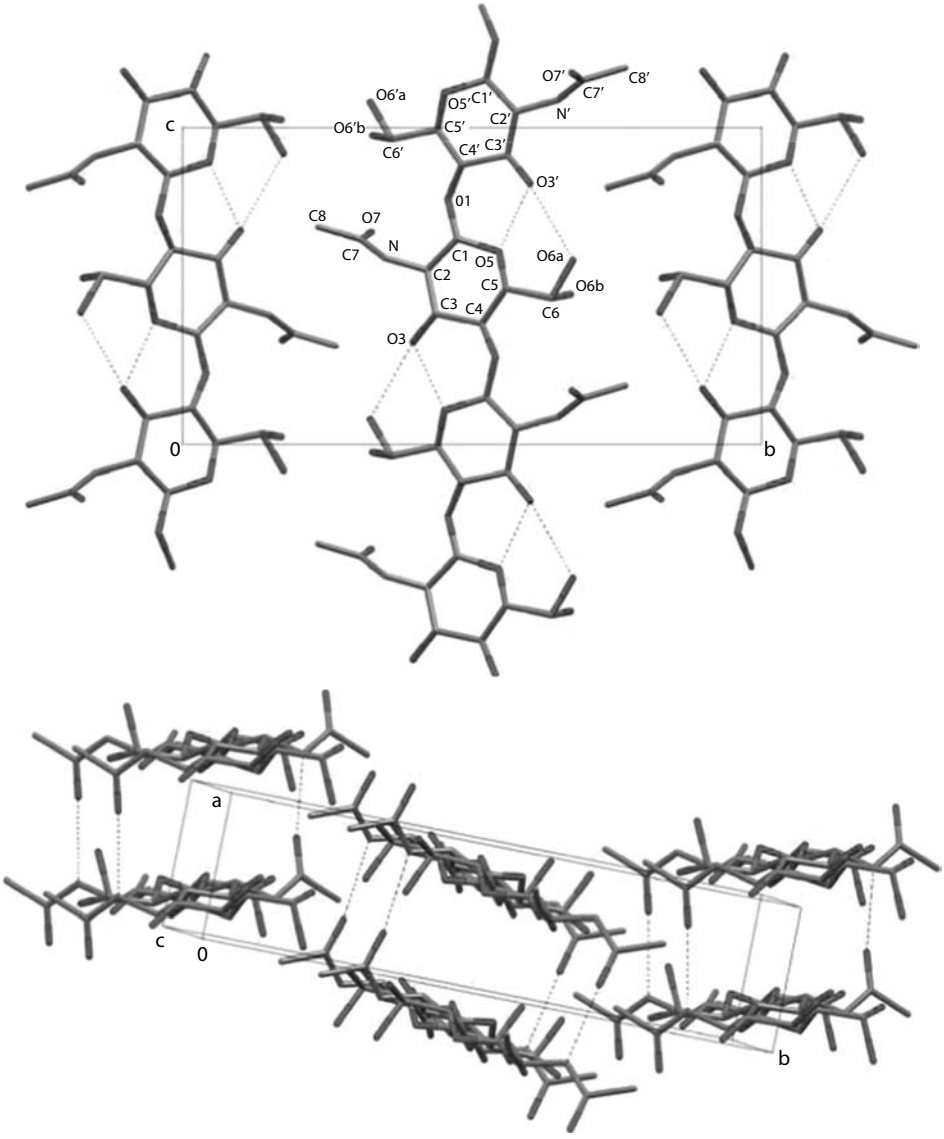

FIGURE 8.5 The crystal packing and labeling scheme for α-chitin. The hydrogen atoms are omitted for clarity. Dashed lines represent hydrogen bonds.

8.2.3 Crystal Structure of β-Chitin

X-ray diffraction data of β-chitin were first indexed for a unit cell containing one chitin residue by Dweltz (1961). Blackwell (1969), by using trial-and-error methods, reexamined the β-chitin structure, which was isolated from pogonophore tubes. Finally, the detailed three-dimensional structure of β-chitin using the rigid-body least-squared refinement techniques was reported by Gardner and Blackwell (1975). Unfortunately, due to numerical errors in the original article, it is not possible to reestablish a reasonable crystal structure of β-chitin using published by Gardner and Blackwell atomic coordinates. Recently an exhaustive search of the crystal structure of β-chitin was carried out by simultaneously optimizing all the structural parameters based on published x-ray diffraction data and stereochemical criteria (Yui et al. 2007). The β-chitin adopts a monoclinic unit cell,

the space group is $P2_1$, and the unit cell contains two sugar residues related by the twofold screw axis. The last crystal structure search of β-chitin confirmed the parallel-up chain polarity. Similar to α-chitin, a strong intermolecular C=O·····H–N hydrogen bond is observed between the chains along the *a*-axis, which allowed stable chain stacking in this direction. In α-chitin, there are also some intersheet hydrogen bonds along the *b*-axis involving the association of the hydroxymethyl groups of adjacent chains. Such a feature is not found in the structure of β-chitin, which is therefore more susceptible than α-chitin to intracrystalline swelling.

8.3 CHITOSAN STRUCTURE IN THE SOLID STATE

8.3.1 General Remarks

The main derivative of chitin is chitosan, produced by the alkaline deacetylation of chitin. Although, a sharp nomenclature border does not exist between chitin and chitosan, the term "chitosan" usually represents copolymers of 2-amino-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose, where the degree of deacetylation is generally more than 60%. Chitosan also occurs naturally in some fungi but its occurrence is much less widespread than that of chitin. Its chemical structure is presented in Figure 8.6. The major investigations of chitosan concern its preparation with varied molecular weights and the degree of deacetylation from chitin. In the solid state, chitosan is a semicrystalline polymer. Despite that, several crystalline polymorphic forms of chitosan have been reported so far in the literature (Clark and Smith 1936, Samuels 1981, Ogawa et al. 1984, 1992, Cartier et al. 1990, Ogawa and Yui 1993, Mazean et al. 1994, Yui et al. 1994, Kawada et al. 1999, Okuyama et al. 1997, 1999). All having an extended twofold helical structure, but differing in packing density and water content. Only "tendon" and "annealed" polymorphs were analyzed very precisely (Okuyama et al. 1997, 1999). Another polymorphs called "L-2," "1-2," form I and II require father investigations. The hydrated "tendon" chitosan can be converted quite easily to the anhydrous crystalline form by annealing (Ogawa 1991) or transformation via chitosan/monocarboxylic salt (Kawada et al. 1999, Okuyama et al. 1999). Crystal structures and lattice parameters for tendon and annealed polymorphs are given in Table 8.2.

8.3.2 Molecular Conformation of Chitosan

Figure 8.7 shows the principal parameters to describe chitosan chain conformation together with atomic labeling scheme. Two torsion angles ϕ (O5–C1–O1–C4′) and ψ (C1–O1–C4′–C5′) define the main chain conformation. τ is the glycosidic bridge angle. The orientation of the O6 atom is defined by the dihedral angle χ (O5–C5–C6–O6). The conformation of O6 usually prefers the

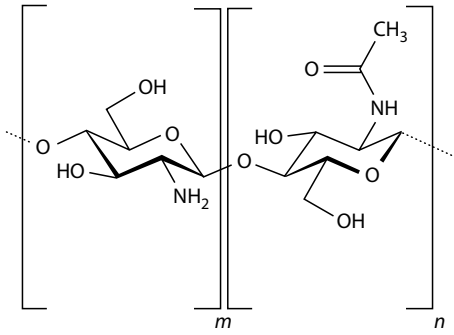

FIGURE 8.6 Chemical structure of chitosan composed of β-(1 → 4)-linked 2-amino-2-deoxy-D-glucopyranose (*m*) and 2-acetamido-2-deoxy-D-glucopyranose (*n*).

TABLE 8.2
Crystallographic Parameters of Different Polymorphs of Chitosan

Chitosan	*a* (Å)	*b* (Å)	*c* (Å) (Fiber Axes)	Space Group	Reference
Tendon	8.9	17.0	10.25	$P2_12_12_1$	Clark and Smith (1937)
	8.95	16.97	10.34	$P2_12_12_1$	Okuyama et al. (1997)
Annealed	8.26	8.50	10.43	$P2_12_12_1$	Okuyama et al. (1999)
	8.28	8.62	10.43	$P2_12_12_1$	Yui et al. (1994)
	8.07	8.44	10.34	$P2_12_12_1$	Mazean et al. (1994)

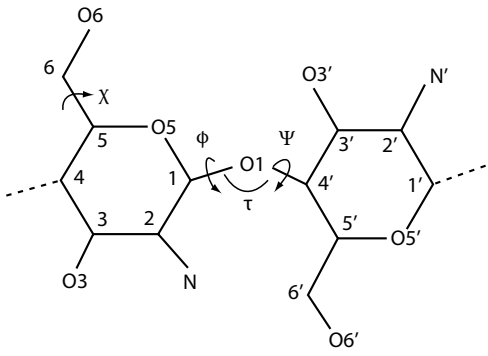

FIGURE 8.7 Definition of atomic labels and chain conformation angles of primary interest (for definition see text). Hydrogen atoms are omitted for clarity.

three staggered positions, each of which is referred to as *gauche–trans* (*gt*), *gauche–gausche* (*gg*), or *trans–gauche* (*tg*) (Sundaralingam 1968, Marchessault and Perez 1979). Typical values of χ are 60°, −60°, 180° for *gt*, *gg*, and *tg*, respectively.

8.3.3 The Crystal Structures of Tendon and Annealed Chitosan

The x-ray fiber diffraction data of chitosan was first derived from the solid-state deacetylated product of a lobster tendon chitin by Clark and Smith (1936). Sixty years later, Okuyama et al. (1997) analyzed the pattern of "tendon chitosan." In the "tendon" polymorph, the chitosan chains and water molecules are packed in an orthorhombic unit cell. Each chitosan chain takes an extended twofold helix with a repeating period of 10.34 Å. A "zigzag" structure is stabilized by an O3·····O5 intramolecular hydrogen bond with close to *gauch–trans* (*gt*) orientation of O6 (χ = 69.6°). The main-chain conformation angles, ϕ and ψ, are −92.1° and −147.0°, respectively. Chitosan chains are packed together in an antiparallel fashion. The up-chain and lower-chain are bounded by N·····O6 hydrogen bonds to form a sheet structure. Neighboring sheets are related by the symmetry of 2_1 axis along the *b* direction. As a result, two independent polymer chains form a repeating unit along the *a*-axis. Water molecules form the column between the sheets and stabilize the crystal structure through hydrogen bonds network. This polymorph is the most abundant. A commercially available chitosan has the same crystalline form although their crystallinity may be different.

The "tendon" chitosan can be easily converted to an anhydrous form, that is, by annealing at about 240°C in water (Ogawa 1991). This polymorph is called "annealed" chitosan. As shown in Figure 8.8 in its crystal, each chitosan chain takes an extended twofold helical symmetry reinforced by the O3·····O5 and weak O3·····O6 hydrogen bonds. The main chain conformation angles ϕ and ψ are −96.1° and −145.5°, respectively. The orientation of O6 has a *gt* conformation with χ = 48,8°. The zigzag structure is similar to that of tendon chitosan, and the chains are also packed in an

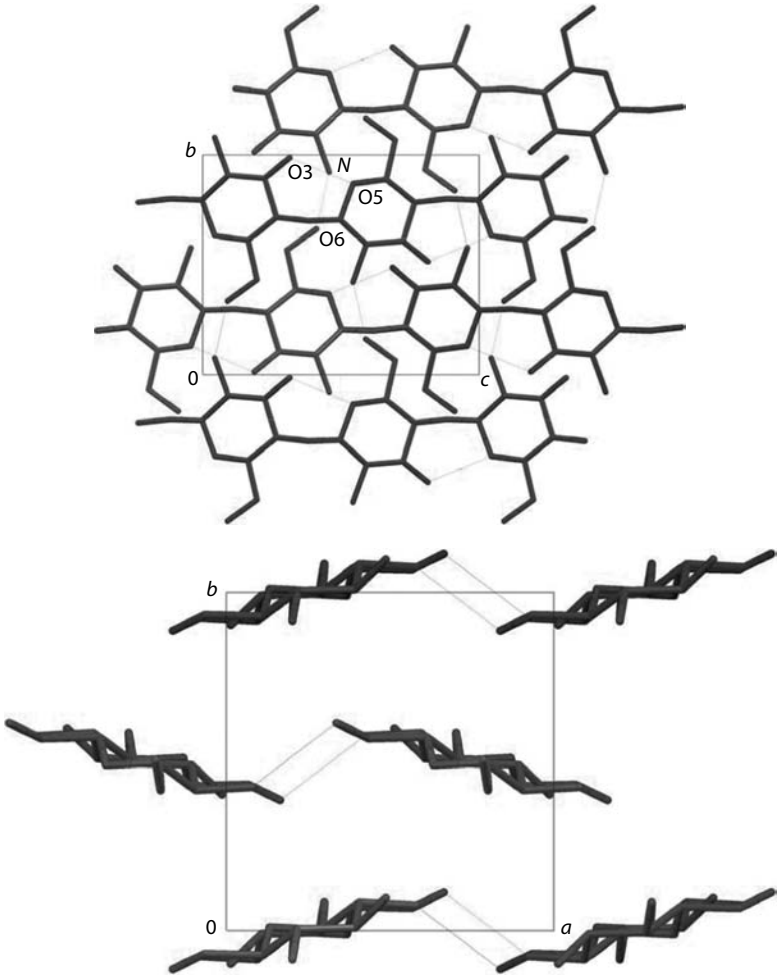

FIGURE 8.8 The crystal packing of the annealed chitosan along *a* (top) and *c* (bottom) axis. The hydrogen atoms are omitted for clarity. Dashed lines represent hydrogen bonds.

antiparallel fashion. However, there are no water molecules in the crystal. Different from tendon polymorph, in annealed chitosan, neighbor chains are bonded by direct hydrogen bonds N2·····O6 to make a sheet, and neighbor sheets are stacked. Similar results were obtained for single crystals of chitosan acquired from fully deacetylated chitin of low molecular weight using an electron diffraction experiment (Mazean et al. 1994). In summary, only the zigzag structure has been reported in the literature for free chitosan so far.

8.4 SALTS AND COMPLEXES

Chitin and chitosan are interesting polysaccharides because of the presence of the amino functionality, which could be suitably modified to impart desired properties (Muzzarelli 1977, Roberts 1992, Hudson and Smith 1998, Peter 2002, Rinaudo 2006). Apart from the amino groups, they have two hydroxyl groups for appropriate chemical modifications. As with cellulose, chitin and chitosan can undergo many of the reactions such as etherification, esterification, and crosslinking (Hon 1996), suggesting these possible chemical modification reactions. Due to the intractability and insolubility of chitin, much attention has been given to chitosan with regard to developing derivatives with well-defined molecular architectures having advanced properties and functions (Muzzarelli 1977,

Dumitriu 1996). A number of authors have reviewed the area emphasizing various aspects of chemical modification of chitosan (Kurita 1996, Muzzarelli 1996, Jenkins and Hudson 2001, Sashiwa and Aiba 2004, Ma et al. 2004, Muzzarelli and Muzzarelli 2005, Jayakumar et al. 2005, 2006, 2007, Prashanth and Tharanathan 2007). In this context, many x-ray diffraction patterns from chitosan derivatives (mainly acid salts and chitosan complexes with metal salts) have been reported so far (Ogawa and Inukai 1987, Ogawa et al. 1996, Kawada et al. 1999, Okuyama et al. 2000a,b, Kawahara et al. 2003, Lertworasirikul et al. 2003, 2004). Chitosan forms water-soluble salts with both inorganic and organic acids. In terms of polymer conformation, they have been classified into four groups depending on the kind of acid, concentration, and temperature used during salt preparation (Table 8.3; Ogawa et al. 2004, Ogawa 2005).

Type I salts are anhydrous, so the backbone chitosan chains retain the extended twofold helix as in the free chitosan. For example, chitosan–hydrogen iodide salt obtained at room temperature crystallizes into form I. Two chitosan chains and four iodide ions are packed in a monoclinic unit cell: a = 9.46 Å; b = 9.79 Å; c = 10.33 Å (fiber axes); β = 105.1°. Each chitosan chain takes an extended twofold helical symmetry with repeating fiber axis lengths. The molecular structure is stabilized by O3·····O5 intermolecular hydrogen bond (Figure 8.9). The main chain conformation angles ϕ and ω are 152.0° and 87.6°, respectively. The glycosidic linkage angle τ is 116.7°, and the O6 atom possesses *gt* conformation. Two adjacent chains are related to each other by the 2_1 symmetry along *b*-direction. The corner chain is oriented up, while the second chain at the center of the *b* axis is oriented down. These two chains are arranged in an antiparallel fashion, and they are linked along the *b* axis by two N2·····O6 hydrogen bonds to form a zigzag sheet piled up along the *a*-direction. The two iodide ions are on the top of the zigzag structure, they stabilize the salt structure similarly as water molecules by forming hydrogen bonds between N2 and O6. The columns of iodide ions also maintain the structure by electrostatic interactions between N2 and iodide ions (Lertworasirikul et al. 2004).

On the contrary, conformational changes of chitosan molecules are present when chitosan creates the salts of types II and III. While tendon chitosan was immersed in a mixture of water solution of acetic acid and isopropanol (Yamamoto et al. 1997), the resulting salt gave a diffraction pattern showing about four times longer fiber axis than that of type I. It was obvious that conformation changes of chitosan have appeared. The salt was called type II (Okuyama et al. 2000a,b, Lertworasirikul et al. 2003). Like in the type I the molecules are arranged in the antiparallel fashion, but the asymmetric unit consists of four glucosamine residues. The conformation of chitosan in these salts is called relaxed 2/1 helix. Another type II salt named type IIa has been found for chitosan hydrogen iodide salt prepared at 4°C (Lertworasirikul et al. 2003). The second crystalline iodide

TABLE 8.3
Structural Diversity of Chitosan Acid Salts

Type	Chitosan Conformation	Acid
I (anhydrous)	An extended twofold helix	HNO_3,[a] HBr, HI,[b] $HClO_4$,[c] L- or D-lactic acid,[b] maleic acid, L-ascorbic acid, D-isoascorbic acid, salicylic acid[d]
II (hydrated)	A relaxed twofold helix	HNO_3,[e] H_2SO_4, HCl, HF, HIO_4,[c] H_3PO_4,[d] L-or D-lactic acid,[d] succinic acid, fumaric acid, L-tartaric acid, monocarboxylic acid (formic acetic, propionic, butyric)
IIa (hydrated)	A 4/1 helix	HI[d]
III (anhydrous)	A 5/3 helix	Salicylic acid,[d] gentisic acid, aspirin[c]

[a] High acid concentration.
[b] Higher temperature.
[c] Solid-state ^{13}C NMR.
[d] Lower temperature.
[e] Low acid concentration.

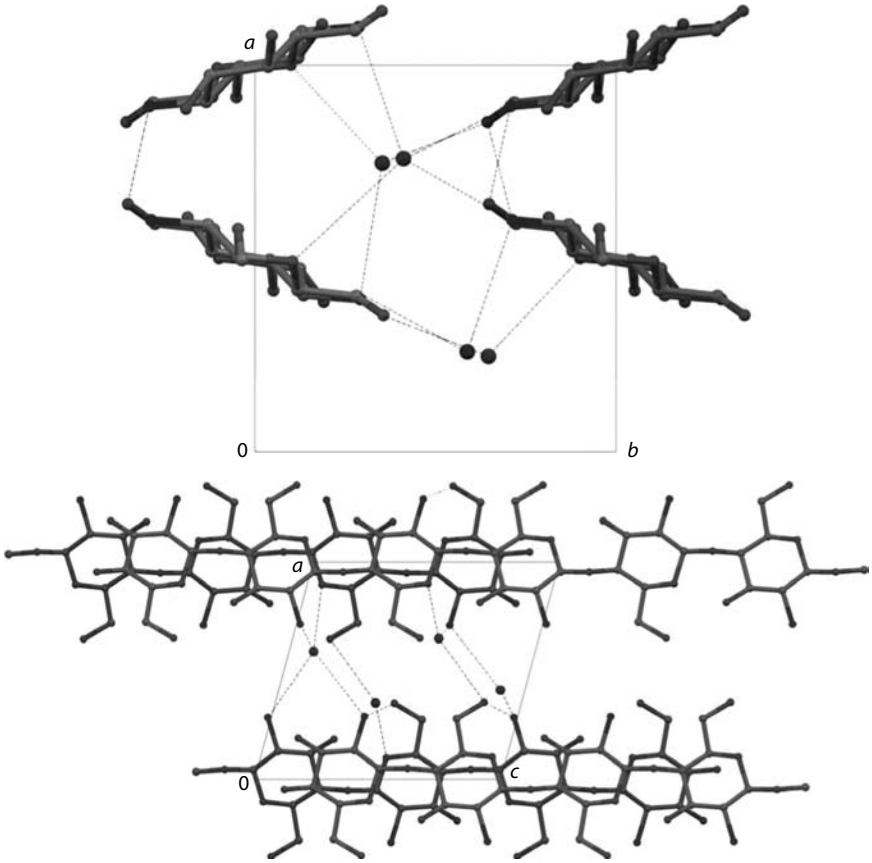

FIGURE 8.9 The crystal packing of chitosan HI type I salt along *c* (top) and *b* (bottom) axis. The hydrogen atoms are omitted for clarity. Dashed lines represent hydrogen bonds.

salt crystallizes in a tetragonal system with unit cell parameters $a = b = 10{,}78$ Å; $c = 40{,}57$ Å (fiber axis). The molecular conformation is a 4/1 helix with an asymmetric unit of glucosamine dimmer residue. Recently a new chitosan conformation called type III salt has been found in the chitosan salt with organic acids having phenyl group (i.e., salicylic and gentisic acids). Their fiber patterns have fiber repeat of 25,5 Å, indicating that the chitosan chain takes a 5/3 helix conformation with asymmetric unit of glucosamine residue (Kawahara et al. 2003).

One of the major applications of chitosan is based on its ability to bind strongly to metal ions. Immersing a tendon chitosan in various metal salt solutions such as Cd^{2+}, Cr^{3+}, Hg^{2+}, $Pb^{2+}Zn^{2+}$, or Cu^{2+} ions give x-ray diffraction patterns where the primary amino group is one of the ligand. All crystals known so far were indexed with the orthorhombic unit cell. The conformational pattern of chitosan chain was identical to the tendon form of chitosan.

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9 Mechanical Properties of Chitosan and Chitosan–Poly(Vinyl Alcohol) Blend Films

Masaru Matsuo, Yumiko Nakano, Teruo Nakashima, and Yuezhen Bin

CONTENTS

9.1 INTRODUCTION

Over the last 10 decades, the global environmental problem has attracted considerable attention of the research community and policymakers, and the development of polymer materials, which are degradable in a natural environment, is a priority among researchers. The production of biodegradable polymers, which are decomposed by microorganisms and photodegradable polymers that are decomposed by sunlight, have been receiving increasing attention.

An ideal biodegradable polymer material is one that can be recycled many times before promptly being decomposed by microorganisms or sunlight, and providing carbon dioxide and water after being disposed of.

Chitin and chitosan belong to a group of natural polymers produced by the shells of crab, shrimp, and lobster, but they have not been utilized effectively. Chitin ranks second to cellulose as the most plentiful organic compound on earth. Almost all of the estimated 10^{10}–10^{11} tons of crab, shrimp, and lobster produced annually could be used as raw materials. Chitin and chitosan are, therefore, very beneficial natural resources when the economical recycling of wastes is considered. Accordingly,

there have been a number of reports (Borynies et al. 1996, Saimoto et al. 1996, Sashiwa et al. 2002, 2003, Ratajska et al. 2003a,b, Zheng et al. 2004) concerning the biodegradation of chitin and chitosan through chemical modification. However, the effect of the biodegradation of these materials buried in the soil (outdoor) must be carefully examined in terms of their effect on the natural environment.

Man-made poly vinyl alcohol (PVA) films and fibers are polymer materials that are known to biodegrade when disposed in the outside environment. This is attributed to the hydrophilic property of PVA. In spite of a number of published papers on PVA, the thermal and mechanical properties remain an unresolved problem because of the difficulty in measuring the much smaller change in crystallinity with respect to temperature in comparison with poly-α-olefin polymers such as polyethylene and polypropylene.

To develop recyclable materials from the viewpoints of environmental protection and ecology, as discussed above, the expansion of mechanical properties of chitosan and chitosan/PVA blend films and the fibers are very important for their utility as industrial materials. However, the maximum draw ratio of chitosan films was only two times under steam and the Young's modulus was ca. 6 GPa, which is slightly lower than that of common industrial materials such as nylon-6, poly(ethylene terephthalate), and polypropylene fibers. This is due to the poor molecular orientation caused by the low draw ratio (two times). To pursue the wider utilities of chitosan, the admixture of chitosan into PVA is carried out to promote an increase in the Young's modulus, since PVA is one of a number of degradable synthetic polymers with good drawability. Blends of chitosan and PVA with good miscibility have been reported to provide good mechanical properties (Miya et al. 1983, Miyashita et al. 1999, Zheng et al. 2001, Castro et al. 2005, Wang et al. 2005), drug release control (Kim et al. 1992, Khoo et al. 2003), and an approach for producing polymeric packaging films for specific purposes (Srinivase et al. 2003).

In several papers (Arvanitoyannis et al. 1997, Chuang et al. 1999, Koyano et al. 2000, Yang et al. 2004), however, the two polymers have been reported to be essentially immiscible. They reported the difficult compatibility by differential scanning calorimetry (DSC), scanning electron microscopy (SEM), Fourier transform infrared spectrum (FTIR), electron-probe microanalyzer, etc. Among them, Koyano et al. (2000) pointed out that the chitosan content is dependent upon the position of the film and chitosan is concentrated on the air-surface side of the films. In another research, however, Arvanitoyannis et al. (1997) reported that the difficulty could be mitigated by adding sorbitol and sucrose as plasticizer.

In this chapter, first, the individual thermal and mechanical properties of chitosan and PVA as-cast films were investigated for as-cast films containing water and perfectly dried films in relation to molecular mobility of PVA chains by using x-ray, DSC, positron annihilation, and viscoelastic measurements. Based on the results, the detailed characteristics of the blends were analyzed as a function of chitosan content in terms of the individual properties of chitosan and PVA. Further analysis of the blend films was carried out for chitosan content on the film surface of drawn films by electron spectroscopy for chemical analysis (ESCA) and water-contact angle experiments.

9.2 EXPERIMENTAL

9.2.1 Preparation Method of Films

9.2.1.1 Chitin Films

One gram of chitin powder produced by Funakoshi Co., Ltd. was dispersed into a mixture containing 50 mL of *N*,*N*-dimethyl acetamide and 50 mL of *N*-methyl-2-pyrolidone, and then 5 g of LiCl was added slowly (Uragami et al. 1981). The solution was mixed for 24 h and then poured into a petri dish. It was then coagulated with 2-propanol for 2 h to form a chitin film. The cast films were washed with water. The film was permeated into hot water at 80°C for 2 h and then fixed by a ring to avoid shrinkage and dried. The amount of solution poured was controlled to create as uniform a film thickness as possible. The actual thickness of the cast films was in the range of 0.18–0.24 mm.

9.2.1.2 Chitosan Films

Chitosan powder used in this research was furnished by Funakoshi Co., Ltd. and had 85% deacetylation property and a viscosity of 5–20 cp. To prepare the chitosan film, 4 g of chitosan was first dispersed into 100 mL of distilled water and stirred for 30 min to make it well infiltrated. Four kinds of acid—acetic acid, formic acid, butyric acid, and propionic acid—were used as solvents (Sakurai et al. 1984). Four milliliters of each solvent was added to the chitosan solution individually and mixed for 24 h and then the solution was cast in a petri dish. The solvent was evaporated at room temperature to form the cast film. Each chitosan film was immersed into 4% of sodium hydroxide for 24 h to neutralize the films and then washed well with distilled water. The resultant films were elongated up to two times of their original size under steam.

9.2.1.3 Chitosan and PVA Blend Films

At first, 2 g chitosan was dispersed in 100 mL distilled water and stirred for 30 min. One milliliter of acetic acid was added into the chitosan aqueous solution, and mixed for 24 h at room temperature. The PVA powder of Nacalai Tesque. Inc. was used as a sample, and possessed a degree of polymerization of 2000% and 98% hydrolysis. The PVA powders were dissolved in distilled water to maintain a 3% solution concentration and stirred for 40 min at 95°C. After, the solution was cooled down to room temperature. The corresponding solutions of chitosan and PVA, respectively, were simultaneously poured into a flask in the desired proportion of chitosan/PVA and stirred for 3–5 h at room temperature. The weight compositions chosen were 100/0 (chitosan homopolymer), 61/39, 50/50, 40/60, 30/70, 22/78, and 0/100 (PVA homopolymer). The mixed solution was poured into a petri-dish and the distilled water was allowed to evaporate to produce the cast film. The resulting film was first immersed into 4% sodium hydroxide for 2 h to neutralize the cast film and then washed well with distilled water. Finally, the film was vacuum-dried for 1 day to remove residual traces of water and then placed inside a desiccator to avoid moisture absorption.

The resultant as-cast films were cut into strips, 60 mm long and 10 mm wide, and then clamped in a manual stretching device in such a way that the length to be drawn was 40 mm. The specimens were placed in an oven at 160°C and elongated manually for a desired number of times after preheating for 5 min. The maximum draw ratios were three times ($\lambda = 3$) for the 40/60, five times ($\lambda = 5$) for the 30/70, seven times ($\lambda = 7$) for the 22/78 and eight times ($\lambda = 8$) for the 0/100. The elongation was done smoothly without any partial breaking and the drawn blend films were transparent. Here, we must emphasize that the uniform mixing of the mixed solution (the chitosan content being higher than 50%) was very difficult and any elongation of the nonuniform resultant blend films was impossible.

9.2.2 Evaluation of Biodegradation

Test specimens of chitin and chitosan films were cut into 5 × 5 cm squares to evaluate their biodegradation properties. For the biodegradation test, they were buried in the soil (outdoors), 10 cm below the surface. Two kinds of soils, paddy soil and red clay, were chosen. The burying period of the test samples was 1, 1.5, and 2 months.

A biodegradation test in a room (indoor test) was also carried out using chitosan-degrading bacteria, which was taken from soil. The isolation of chitosan-degrading bacteria was conducted according to the method described previously (Toyoda et al. 1993, Matsuda et al. 2001). The indoor biodegradation specimens were tested by cultivating for 0, 0.5, 1, and 1.5 months in a M9 culture medium (0.6 g $Na_2HPO_4 \cdot 7H_2O$, 0.3 g KH_2PO_4, 0.05 g NaCl, 0.1 g NH_4Cl, 0.02 g $CaCl_2 \cdot 2H_2O$, and 0.12 g $MgSO_4 \cdot 7H_2O$ in 1 L of water), supplemented with colloidal chitin or colloidal chitosan (Toyoda et al. 1993).

The abstraction of the buried-disintegration bacteria from soil was performed using the following process. At first, 1 g of soil around the test films buried outdoors was extracted as a test sample and added to a 100 mL physiological salt solution to form suspension. The suspension was vibrated

violently for 10 min and filtered out. A M9/agar culture medium supplemented with colloidal chitosan was added to the filtrate and then cultivated at 30°C for 7 days, using agar plate dilution method. The bacteria, which were taken from the distinct halo formed around the grown colony after cultivation, were chosen as chitosan decomposition bacteria. The decomposition bacteria were identified as *Sphingobacterium multivorum* through the cloning measurement of the chitosanase gene (Matsuda et al. 2001). The colloidal chitin was decomposed with the above bacteria. The biodegradation of chitin and chitosan films was evaluated by weight loss and the structural change was observed by SEM.

9.2.3 Measurements of Characteristics of Chitosan, Chitosan/PVA Blend, and PVA Films

The structures of the crystallites of chitosan, chitosan/PVA blend, and PVA films were also estimated using a 12 kW rotating-anode x-ray generator (Rigaku RDA-rA) operated at 150 mA and 40 kV. The x-ray beam using CuKα radiation was monochromatized with a curved graphite monochromator. The diameter of the x-ray cell tube was 1 mm. WAXD intensity measurements were carried out using two methods. The first was a step-scanning method with a step interval of 0.1° taken at each fixed time of 40 s, from 10° to 30° at 25°C, and the second, a curved position-sensitive proportional counter (PSPC) to estimate the change in diffraction intensity distribution as a function of twice the Bragg angle simultaneously at a temperature ranging from −40°C to 50°C.

Positron annihilation experiments were conducted with a conventional fast-fast coincidence system, having a time resolution of 300 ps full width at half-maximum (FWHM). The positron annihilation spectrometer was composed of two plastic scintillation detectors (40 mm diameter × 40 mm Pilot-U mounted on a Hamamatsu H1949 photomultiplier), two differential constant-fraction (ORTEC 583) discriminators (one for the start signals from 1.27 MeV γ-rays, and the other for the stop signals from 0.511 MeV annihilation γ-rays), a time-to-amplitude converter (ORTEC 4570), and a multichannel analyzer with a 1024 conversion gain (SEIKO 7800). A positron source was prepared by depositing ca. 1.1 MBq (30 μ Ci) of aqueous ^{22}NaCl on a Kapton foil of 7 μm thickness and 10 × 10 mm area. After drying, the foil was covered with a second foil of similar size, and the edges of the two foils glued together with epoxy resin. The source was further sealed in a 3 μm Mylar foil and then sandwiched by two identical samples for positron annihilation measurements. The spot diameter of the ^{22}Na source was ca. 2 mm. During the measurements, the samples were kept in a vacuum cell in which the temperature was controlled. Spectra were recorded every hour, and about 1–2 million events were stored in each spectrum. The detailed procedure has been described elsewhere (Ma et al. 2003).

The densities of all the chitosan/PVA blend films were measured using a density gradient tube with *n*-heptane and carbon tetrachloride as the medium at 20°C. Since the density of the blend film was dependent on a complete absence of moisture within the film, great care was taken to remove moisture of the film. The samples were cut into fragments, washed through an ultrasonic treatment in ethanol, and then dried in vacuum for 1 day prior to measuring the density.

The thermal behavior of the film was estimated in terms of its melting endotherm as determined by differential scanning calorimetry (DSC). The DSC was performed using a DSC6200 (SII EXSTAR 6000) with 10 mg samples in standard aluminum pans. The samples were heated at a constant rate of 5°C/min under nitrogen. The measurements were done in the temperature range from −130°C to 250°C.

The stress–strain curves were recorded to obtain Young's modulus and tensile strength using a tensile tester (TENSILON/STM-H-500BP) at a cross-head speed of 2 mm/min.

The complex dynamic tensile moduli were measured at a frequency of 10 Hz over the temperature ranging from −150°C to 230°C–300°C by using a viscoelastic spectrometer (VES-R, Iwamoto Machine Co. Ltd.). The length of the specimen between the spectrometer jaws was 30 mm, and the width was ca. 5 mm. The measurements were described in detail elsewhere (Matsuo et al. 1988, 2003).

The surface of the chitosan/PVA blend film was analyzed by electron spectroscopy for chemical analysis (ESCA-850, Shimadzu). The sample was irradiated with monochromated MgKα x-rays (8 kV, 30 mA) and the scanning speed was 0.05 V/s.

Water-contact angle experiments were performed using a Kyowa Interface Science Instrument, CA-X at room temperature (Okuno et al. 2005).

9.3 RESULTS AND DISCUSSION

9.3.1 Characterization of Chitin and Chitosan Films Prepared by Several Kinds of Solvents

Table 9.1 summarizes the characteristics of chitin and chitosan films in an undrawn state. The acidity of the solutions for preparing the films was different. The formic acid solution had the highest acidity with pH 3.8, and the other three kinds of solutions showed almost the same acidity with pH values in the range of 4.0–4.4. The water content in chitosan film was 8.9%–16.2%, which is almost equal to the absorbency of natural fibers and much higher than that of synthetic fibers such as polyethylene and poly(ethylene terephthalate). The densities and tensile strength for chitin films were slightly lower than those of chitosan films. The tensile strength was 0.14–0.16 GPa and Young's modulus was 2.39–2.68 GPa for the chitosan films prepared with four kinds of solvents. This means that there was no great difference in the densities and tensile strengths for the chitosan films prepared with different solvents. In contrast, Young's modulus obtained as the average of the five measurements was very sensitive to the moisture content, indicating the molecular mobility of chitosan chains.

The tensile strength is not significantly different, in spite of the different moisture contents. On the other hand, Young's modulus measured by a tensile tester was lower than the value obtained from the results of storage modulus (E') at room temperature as shown in Figure 9.1. It is attributed to the fact that the film measured by the tensile tester was not heat-treated, while the samples for viscoelastic measurement were preheated at 120°C for 2 h and the measurements were taken in the absence of water. The value of E' in the lower temperature side is largely independent of the draw ratio, but the value of the drawn film was higher on the higher temperature side. Such a small influence of E' on the lower temperature side is due to the drastic increase in the rigidity of chitosan chains themselves and the effect is superior to chain orientation. The loss modulus E'' shows two or three dispersion peaks and the profiles of undrawn films are very sensitive to the solvents used in the preparation of cast films. By contrast, the profile of the drawn films shows a similar tendency. Namely, the peak appeared around −100°C, which is associated with the local motion of amorphous chain segments. The peak appeared at around 100°C and the amorphous chain segments are thought to have large mobility due to the plasticity induced by water.

The weight loss of samples, prepared by acetic acid, buried in soil is listed in Table 9.2. The weight loss of chitin films buried in red clay reached 83.8%, 99.0%, and 100% after 1, 1.5, and 2 months,

TABLE 9.1
Characteristics of Undrawn Chitin and Chitosan Films

Films	Solvents	pH of Solution	Moisture Content (%)	Density (g/cm³)	Tensile Strength (GPa)	Young's Modulus (GPa)
Chitin	—	4.8	18.7	1.400	0.10	1.87
Chitosan	Formic acid	3.8	9.5	1.422	0.14	2.68
	Acetic acid	4.0	16.2	1.420	0.16	2.39
	Propionic acid	4.4	12.8	1.424	0.16	2.53
	Butyric acid	4.3	8.9	1.423	0.14	2.61

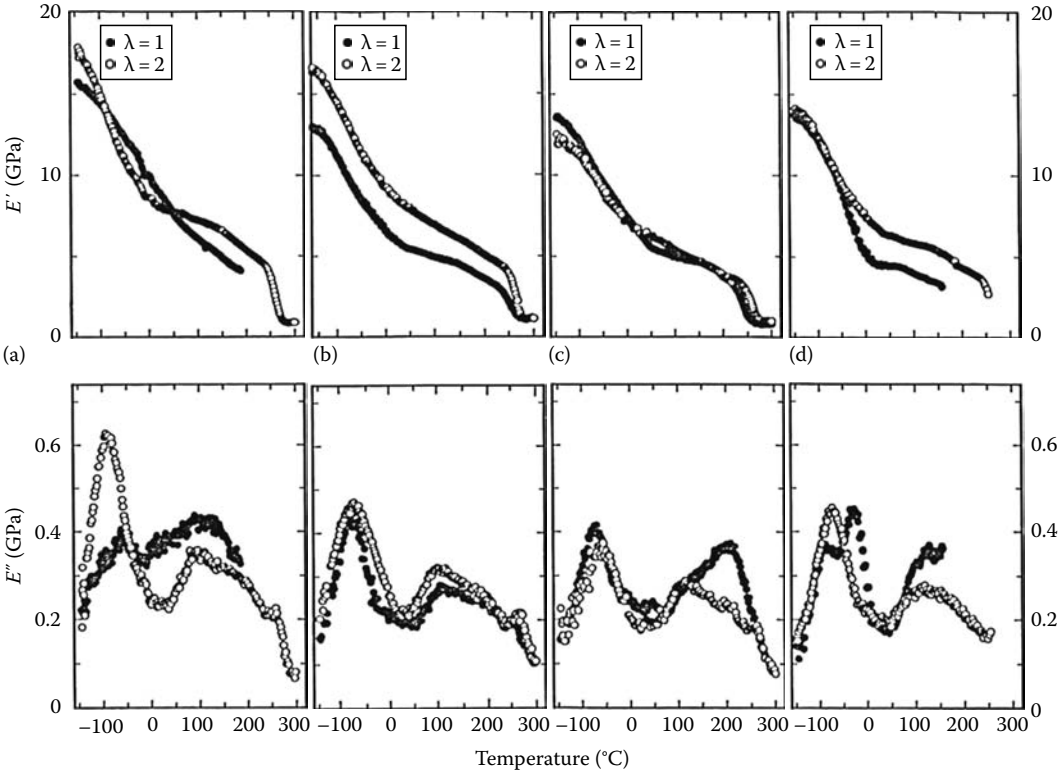

FIGURE 9.1 Temperature dependence of the storage and loss moduli of chitosan films with the indicated draw ratios. The films were heat-treated at 120°C for 2 h. Solvents were (a) acetic acid, (b) formic acid, (c) butyric acid, and (d) propionic acid.

TABLE 9.2
Weight Losses of the Films Buried in Red Clay and Paddy Soil for the Indicated Periods

Soil Type	Burying Period (Months)	Weight Loss (%)	
		Chitin Film	Chitosan Film
Red clay	0	0	0
	1	83.8	79.2
	1.5	99.0	98.9
	2	100.0	100.0
Paddy soil	0	0	0
	1	99.5	83.2
	1.5	100.0	99.1
	2	100.0	100.0

respectively. Namely, the chitin film was degraded perfectly after burying for 2 months. On the other hand, the weight loss of chitin films buried in paddy soils was 99.5% after 1 month and 100% after 1.5 and 2 months, indicating a higher rate of biodegradation. In the case of chitosan films, the weight loss of films was 79.2%, 98.9%, and 100% after burying in red clay for 1, 1.5, and 2 months, respectively, and the corresponding weight loss was 83.2%, 99.1%, and 100% in the case of burying

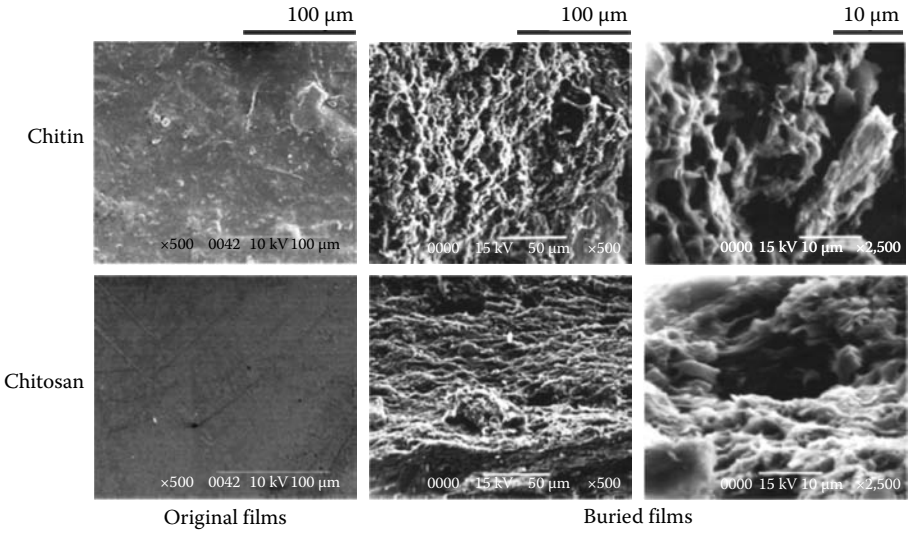

FIGURE 9.2 Scanning electron micrographs of the films buried for 1 month in red clay.

in paddy soils for 1, 1.5, and 2 months. The same tendency was confirmed for chitin film where a perfect decomposition was obtained by burying the films in about 1.5 months. This suggested that the decomposition rate of chitin films was a 5%–6% higher than that of chitosan films when buried in red clay and paddy soil.

Following biodegradation measurement results (Nakashima et al. 2002a,b), the weight losses of films buried in paddy soils were higher than those of films buried in red clay, showing the same tendency as obtained for polyethylene–starch composites and the SEM observation revealed that the decomposition of composite films only occurred in red clay where many holes and cracks were observed not only in the starch component but also in polyethylene. For chitin and chitosan films, however, large holes were observed by SEM for the samples, buried in both red clay and paddy soils, as shown in Figure 9.2. It suggested that the number of bacteria with high biodegradability such as the chitosanase gene in paddy soil is higher than that in red clay and that the chitin films were decomposed into small blocks during biodegradation in soils.

Weight loss of the samples in the indoor test by using the chitosan-degrading bacteria *Sphingobacterium multivorum* is listed in Table 9.3. The weight loss of chitin films was 68.8%, 95.0%, and 100% after cultivating for 0.5, 1, and 1.5 months, respectively. In the case of biodegradation of chitosan films after cultivating for 0.5, 1, and 1.5 months, the corresponding weight loss was 18.0%, 35.4%, and 100% for the films prepared with formic acid; 46.3%, 91.6%, and 100% for

TABLE 9.3
Weight Losses of the Film Samples by Using Incubated Chitosan-Degradation Bacteria (*Sphingobacterium multivorum*)

	Weight Loss (%)				
		Chitosan Film			
Incubation Period (Months)	Chitin Film	Formic Acid	Acetic Acid	Propionic Acid	Butyric Acid
0	0	0	0	0	0
0.5	68.8	18.0	46.3	37.1	10.1
1	95.0	35.4	91.6	77.6	30.7
1.5	100.0	100.0	100.0	100.0	100.0

the films prepared with acetic acid; 37.1%, 77.6%, and 100% for the films prepared with propionic acid; and 10.1%, 30.7%, and 100% for the films prepared with butyric acid. The result of the indoor test shows a similar tendency as that of the outdoor test when the experiment was carried out by using chitosan-degrading bacteria separated from soil. This means that the biodegradation rate of chitin films is faster than that for chitosan films. All chitosan films prepared with four kinds of acid solution were decomposed perfectly after cultivating for 1.5 months. However, the weight loss of the chitosan film was dependent upon the solvents used for the cast. Weight loss of chitosan samples prepared with acetic acid was the highest among the films prepared, followed in order by propionic acid, formic acid, and butyric acid. The weight losses of the chitosan films prepared with acetic acid and propionic acid were about 2.5 times higher than those of the samples prepared with formic acid and butyric acid, indicating a similar tendency.

9.3.2 Characterization of Chitin and Chitosan–Poly(Vinyl Alcohol) Blend Films

As discussed above, the results of a series of experiments carried out both outdoor and indoor indicate that chitin and chitosan are degradable polymers and very beneficial natural resources. But the elongation of chitin was impossible and the maximum draw ratio of chitosan film was two times. To extend further utilities of chitosan as industrial materials, their poor mechanical properties must be improved. As one of approaches to increase mechanical properties, drawing of chitosan and PVA blend films are carried out, since PVA is one of synthetic polymers with degradation and easy drawability.

Figure 9.3 shows the x-ray diffraction intensity curves from chitosan, PVA and four chitosan/PVA blend films (61/39, 50/50, 40/60, 22/78) measured at 25°C, respectively. The curves were drawn in the range of twice the Bragg angle from 16° to 24°. The open circles show the overlapped experimental intensity. Regarding the preliminary experiments, the temperature dependence of the x-ray intensity distribution was measured at 25°C, 60°C, 100°C, 140°C, and 180°C for the undrawn

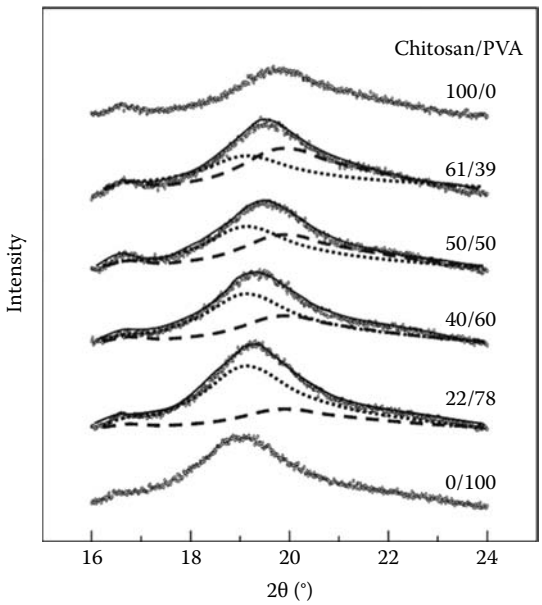

FIGURE 9.3 WAXD intensity curves of chitiosan (100/0), chitosan /PVA blend (61/39, 50/50, 40/60, 22/78) and PVA (0/100) films observed at 25°C. - - - - - - diffraction from chitosan, ... diffraction from PVA, and ———— diffraction form the total. (From Nakano, Y. et al., *Macromol. Symp.*, 258, 63, 2007. With permission.)

chitosan and PVA films, in which the diffraction peaks associated with the chitosan and PVA crystallites appeared at the same diffraction angle independent of the measured temperature. This indicates that thermal crystallization does not occur for chitosan and PVA films due to molecular motion at elevated temperature, which is quite different from the thermal behaviors of certain poly-α-olefins such as polyethylene and polypropylene. Such poor chain mobility is thought to be due to the presence of strong intra- and interhydrogen bonds between the PVA chains. The diffraction peaks from the (110), ($1\bar{1}0$), and (200) planes of the PVA crystallites were overlapped because of low crystallinity. The diffraction peak associated with chitosan was also very weak, indicating low crystallinity. The diffraction intensity profile of the chitosan and PVA crystallites within the blends also showed a very broad overlapped peak. According to Sakurai et al. (1984), the crystal units of chitosan were reported to be dependent upon the dissolved solvents used to prepare the films. The present chitosan film prepared from distilled water containing acetic acid provided a very broad peak profile.

The rough and fine dotted curves obtained by the peak separation shown in Figure 9.3 belong to the diffraction intensity distributions from the chitosan and PVA crystallites, respectively. The peak separation was done by assuming symmetrical profiles for each diffraction curve from chitosan and PVA crystallites. The fitting of the overlapped peak profiles observed for the chitosan and PVA crystallites within each blend film were initially given as a volume fraction ratio of chitosan/PVA calculated from the weight composition, and the volume fraction given as a parameter was slightly changed till the best fitting was achieved. The solid curve, which is the summation of the two separated curves, is in good agreement with the experimental results (open circles). The peaks appearing at 19.3° and 20.2° belong to the chitosan and PVA crystallites, respectively, and any observed peak shift did not occur as a result of changes in the chitosan/PVA compositions. This indicates that blending of chitosan and PVA had no effect on the crystallization of the two polymers. Namely, the observed result represented by open circles shows the simple overlapped peak from the individual chitosan and PVA crystallites. This means that the crystallization of chitosan and PVA occurred independently under evaporation of the solvent. Actually, the densities of the blend films increased linearly with increasing chitosan content (Nakano et al. 2007). Furthermore, we also have to emphasize that x-ray diffraction curves at 130°C were perfectly the same as the profile at 25°C, which indicated no thermal crystallization of PVA.

Figure 9.4 shows the DSC curves of original as-cast chitosan, chitosan/PVA blend, and PVA films measured in the temperature range from −130°C to 250°C. The DSC curve of the PVA film (0/100) shows an endotherm peak around 223°C corresponding to the apparent melting point of the PVA crystallites, where the peak became smaller with decreasing PVA content. Here, it should be noted that the peak position (ca. 223°C) shifted to higher temperature by admixing with chitosan and the shifted peak position (ca. 228°C) was maintained at the same temperature independent of the chitosan content. Judging from the absence of the melting peak of chitosan, the peak shift for the blends is thought to be due to the appearance of the slightly modified PVA crystal unit by admixing with chitosan. This phenomenon must be carefully considered in relation to the miscibility of the chitosan and PVA chains. In the established papers for chitosan and PVA blend films (Koyano et al. 2000, Yang et al. 2004, Wang et al. 2005), the main endothermic peak of PVA appearing at ca. 223°C reported peak shift to lower temperatures. The appearance of the modified crystal, however, cannot be discussed with respect to the present specimens, because the modified peak could not be confirmed by x-ray diffraction intensity curves showing the very broad overlapped peak of the chitosan and PVA crystallites (see Figure 9.3).

Here, it may be noted that the large broad peak of the chitosan film (100/0) formed at lower temperature (100°C–150°C) in Figure 9.4 becomes smaller and is shifted to higher temperature with increasing PVA content. Judging from the temperature dependence of the x-ray diffraction curves shown in Figure 9.3, it may be expected that this endothermic peak of each specimen is obviously independent of melting of a number of small unstable PVA crystallites and is related to the large movement of amorphous chains with pyranose rings. If this is the case, the broad peak shift to

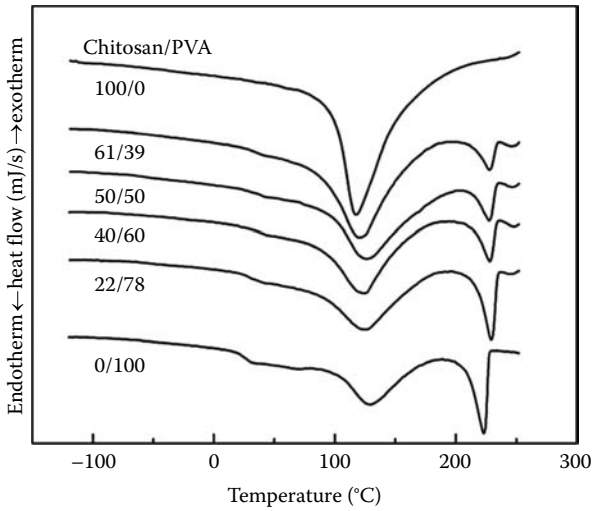

FIGURE 9.4 DSC curves of chitosan (100/0), chitosan/PVA blend (61/39, 50/50, 40/60, 22/78) and PVA (0/100) films observed in the temperature range from –130°C to 250°C. (From Nakano, Y. et al., *Macromol. Symp.*, 258, 63, 2007. With permission.)

higher temperature with increasing PVA content is thought to be due to the comobility of chitosan and PVA amorphous chains associated with the miscibility of the two amorphous chain segments.

In spite of this active chain mobility, any thermal crystallization of the chitosan and PVA chains did not occur as shown in Figure 9.4. According to the established results by Chuang et al. (1999) and Yang et al. (2004), the appearance of a large broad peak at 110°C–130°C was attributed to the influence of water molecules on the chain mobility. To check this, more detailed DSC measurements were carried out for the heating cycles.

Figure 9.5 shows DSC curves for chitosan (100/0), chitosan/PVA blend (22/78), and PVA (0/100) films, in which curves (a) and (b) in each column correspond to the first and second heating runs, respectively. The first heating run was conducted up to 160°C lower than the endothermic peak (ca. 225°C) to avoid chain scission (depolymerization) and carbonization and then cooled to 25°C. No exothermic peak was observed during the first cooling process. After heating above 250°C during the second run, the exothermic peak for chitosan film disappeared, while peaks for PVA and the blend films appeared. The second heating run provided no broad endothermic peak at low temperature (120°C). Accordingly, two possibilities for this phenomenon can be arisen: (1) The broad endothermic peak by the first heating run is related to the active mobility of the amorphous chain segments caused by the presence of small amounts of water molecules, which play an important role as a plasticizer by disruption of the interchain hydrogen bonds between the chitosan chains, where most of the water molecules within the amorphous region were evaporated by the heating up to 160°C. (2) The broad peak is only due to the evaporation of bounded water containing the specimens by the first heating up to 160°C. Of course, the water evaporation was confirmed by TGA measurements, in which the weight loss occurred drastically during the first heating run up to 160°C, and no change in weight loss was confirmed during the second heating because of the evaporation of small amount of water molecules. The question shall be discussed later together with the mechanical properties.

9.3.3 Mechanical Properties

Figure 9.6 shows the temperature dependence of the storage modulus (E') and loss modulus (E'') at a frequency of 10 Hz for chitosan (100/0), chitosan/PVA blend (40/60 and 22/78), and PVA (0/100) films. The results for the second heating run were shown as figures. The first heating run was

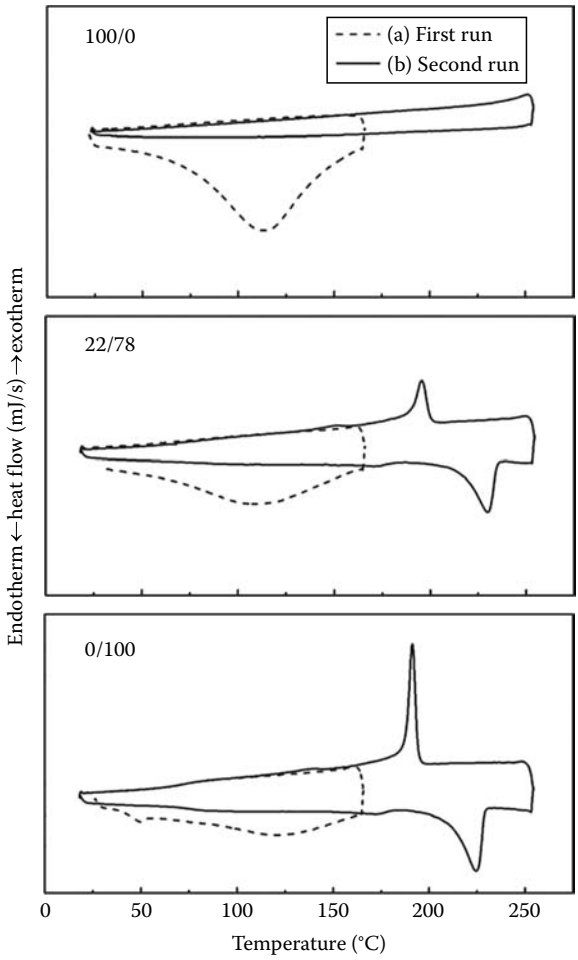

FIGURE 9.5 DSC curves of chitosan (100/0), chitosan/PVA blend (22/78), and PVA (0/100) films measured under repeated heating and cooling processes: (a) the first heating from 25°C to 160°C and (b) the second heating from 25°C to 250°C. (From Nakano, Y. et al., *Macromol. Symp.*, 258, 63, 2007. With permission.)

performed from −150°C to 160°C, and the sample cooling was done from 160°C to room temperature. After, each specimen was cooled down to −150°C in liquid nitrogen and then heated again up to 300°C for chitosan film and 230°C for chitosan–PVA blend films. Interestingly, the E' and E'' curves for the first heating run up to 160°C almost overlapped with the curves for the second heating run, indicating that the E' and E'' of original as-cast chitosan, chitosan/PVA blend, and PVA films vacuum-dried at room temperature are hardly affected by the mobility of the amorphous chain segments of chitosan and PVA due to the active mobility of water molecules detected clearly by the DSC measurements (see Figure 9.5). Namely, it is probably reasonable to consider that the temperature dependence of the E' and E'' are strongly dependent upon the intrinsic mechanical dispersions of the chitosan and PVA films, but the present condition, however, is different from the conditions (Ogura et al. 1980, Pizzoli et al. 1991, Miyashita et al. 1995, Sakurai et al. 2000) reported for the chitosan films containing moisture. Accordingly, the broad peak shown in Figure 9.5 is probably thought to be due to only evaporation of small amount of bounded water connected tightly with hydrogen groups of amorphous chain segments of chitosan and PVA chains.

A detailed observation reveals that the decrease in E' for the chitosan film tended to level off in the temperature range 0°C–50°C, although E' for the PVA and blend films decreased with

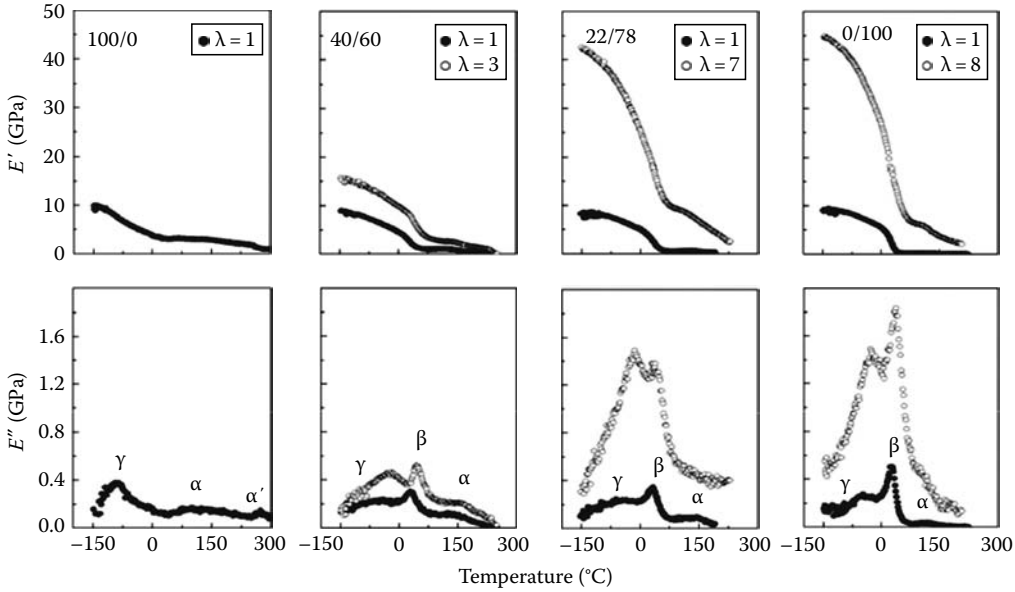

FIGURE 9.6 Temperature dependence of the storage modulus (E') and the loss modulus (E'') at a frequency of 10 Hz for chitosan (100/0), chitosan/PVA blend (40/60 and 22/78), and PVA (0/100) films. (From Nakano, Y. et al., *Macromol. Symp.*, 258, 63, 2007. With permission.)

increasing temperature. The E'' curve of the chitosan film exhibits three peaks. The first peak appearing around −100°C is termed the γ dispersion peak, while the second peak around 120°C is termed the α dispersion peak. The third peak termed as the α′ dispersion peak appeared at ca. 275°C. The γ dispersion peak is thought to be due to the motions of different groups with different steric or energetic interactions. The hindered rotation of the pyranose rings around the ether bond below the glass transition temperature provides one of the possibilities. The second (α) dispersion peak is very broad. Judging from the DSC results discussed already, the α dispersion is obviously not attributed to the crystalline region, but to the amorphous region. According to the results of Sakurai et al. (2000) for the chitosan film, the peak represented as tan δ appeared around 155°C under the first heating process, and the peak appeared as a shoulder in the range from 200°C to 210°C under the second heating run. Furthermore, they also pointed out that the glass transition temperature of the chitosan film is 203°C by DSC measurements. Further studies on chitosan were reported by Pizzoli et al. (1991), Ogura et al. (1980), and Miyashita et al. (1995), and most of their discussions were focused on the mechanical properties and glass temperature of the chitosan films in both wet and dry states. They also reported that the glass temperature of chitosan is ca. 200°C, while the E' and E'' values are sensitive to the moisture in the film.

In the present work, the endothermic peak of chitosan in Figures 9.4 and 9.5 is related to the α dispersion peak of E'' in Figure 9.6, indicating that the α dispersion is attributed to the active mobility of the amorphous chains. Accordingly, the active amorphous chain mobility associated with the α dispersion is thought to be due to the disruption of the interchain hydrogen bonds by the active mobility of the water molecules remaining between the chitosan chains in spite of the first heating run up to 160°C.

In contrast, for the undrawn PVA film, there were three different peaks from the high-temperature side termed the α, β, and γ dispersion peaks. The E' value of the PVA film decreased drastically in the temperature range from 5°C to 50°C. This temperature range is much lower than the endothermic peak of the PVA film (see Figure 9.4). The analysis of this peak shall be discussed in relation to the positron annihilation results later.

It is seen that E'' for undrawn PVA film shows broad and sharp peaks around −35°C and 30°C, respectively, which are termed as the γ and β peaks, respectively. Similar peaks appearing below 0°C were observed by Nishio et al. (1988), and in their study, other peaks located at about 80°C and 35°C were reported as the dispersions due to relaxations in the amorphous PVA regions, and the dispersions observed above 100°C were due to the crystalline relaxations of PVA.

As for the drawn films, E' of the 22/78 ($\lambda = 7$) and the 0/100 ($\lambda = 8$) decreased drastically with increasing temperature up to 50°C. E'' provided a large peak at around 30°C, indicating that E' and E'' are strongly affected by the macro-Brownian movement of amorphous chain segments of PVA associated with the β dispersion. Certainly, at the frozen state of the macro-Brownian movement, the preferential orientation of crystal and amorphous chains, with respect to the stretching direction offered high storage modulus.

In accordance with the undrawn PVA film prepared from a (70/30) mixture of dimethyl sulfoxide (Me_2SO) and water, the corresponding γ and β peaks appeared at −70°C and −10°C, respectively, and the peaks were much broader (Bin et al. 2001). This indicates that the fluctuation in the chain arrangements in the amorphous phase for the PVA film prepared from aqueous solution is larger than that for the PVA film prepared from a Me_2SO/water mixture. Thus it may be expected that the oriented amorphous chains in the present specimen are isolated independently and do not form the aggregation similar to para-crystallites with interchain hydrogen bonds between PVA chains.

Further analysis of the chitosan/PVA blend film was done in terms of the simple additivity of the complex moduli of chitosan and PVA. Theoretical calculations were carried out as a function of chitosan content by using the three-dimensional model in Figure 9.7 (Matsuo et al. 1980, 1990, 2001), in which the chitosan layers are surrounded by a PVA plane, so that the strains of the two phases at the boundary in three directions are identical. The parameters δ, υ, and μ correspond to the fraction length of chitosan in the three directions, and the volume fraction of chitosan is given as δυμ. By using the model system, the complex moduli of the blend may be given by

$$E^* = \frac{E_2^*\left\{E_1^*(1-\delta) + \delta E_2^*(1-\upsilon\mu) + \delta\upsilon\mu E_1^*\right\}}{E_1^*(1-\delta) + \delta E_2^*} \tag{9.1}$$

In Equation 9.1, E_1^* and E_2^* are the complex moduli of chitosan and PVA films in the non-deformed state. The derivation of Equation 9.1 is given in Appendix 9.A.

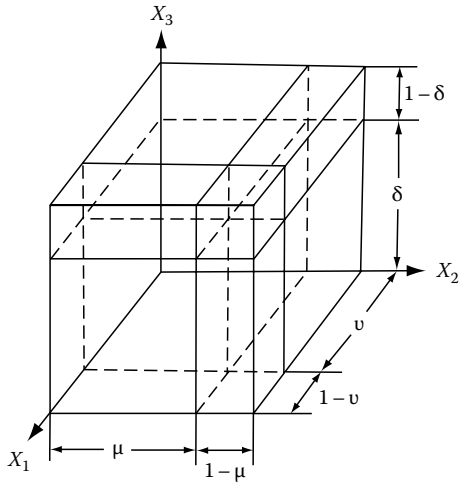

FIGURE 9.7 A composite model in which the oriented crystallites are surrounded by an anisotropic amorphous phase. (From Matsuo, M. et al., *Macromolecules*, 23, 3266, 1990. With permission.)

The values of the three parameter δ, υ, and μ are unknown factors, while the volume fraction δυμ is known. It is generally reasonable to assume that δ = υ = μ for an undrawn film (Matsuo et al. 1980, 1990, 2001). In this case, Equation 9.1 may be rewritten as

$$E^* = \frac{E_2^*\left\{E_1^*(1-\delta)+\delta E_2^*(1-\delta^2)+\delta^3 E_1^*\right\}}{E_1^*(1-\delta)+\delta E_2^*} \tag{9.2}$$

As shown in Figure 9.8, the theoretical solid curves are in good agreement with the experimental results indicating that the two complex moduli of chitosan and PVA satisfy the simple additivity of Equation 9.2. This suggests that the crystallization of the PVA chains is almost independent of the chitosan content, and this concept also supports the linear relationship for the density of the blend film with respect to the chitosan content reported elsewhere (Nakano et al. 2007). Further, detailed analysis of x-ray diffraction peaks was done for chitosan, chitosan/PVA blend (22/78), and PVA films in the temperature range from −40°C to 50°C. The results reported that the β dispersion peak of E'' around 30°C is independent of thermal crystallization. Accordingly, the β dispersion peak is related to the active mobility of the PVA amorphous chains associated with macro-Brownian motion and this concept supports the drastic decrease of the corresponding E'.

An increase in the average free volume holes can be clearly monitored by positron annihilation. Positron annihilation is one of the useful techniques to investigate relaxation characteristics of polymers. Positrons emitted from ^{22}Na induce radiation effect on polymer samples, and then resultant electrons are trapped in a shallow potential, which are formed at low temperature far below the glass temperature (T_g). The increase in the number of these trapped electrons is observed as an increase in the intensity (I_3) of the long-lived component of *ortho*-positronium (*o*-Ps).

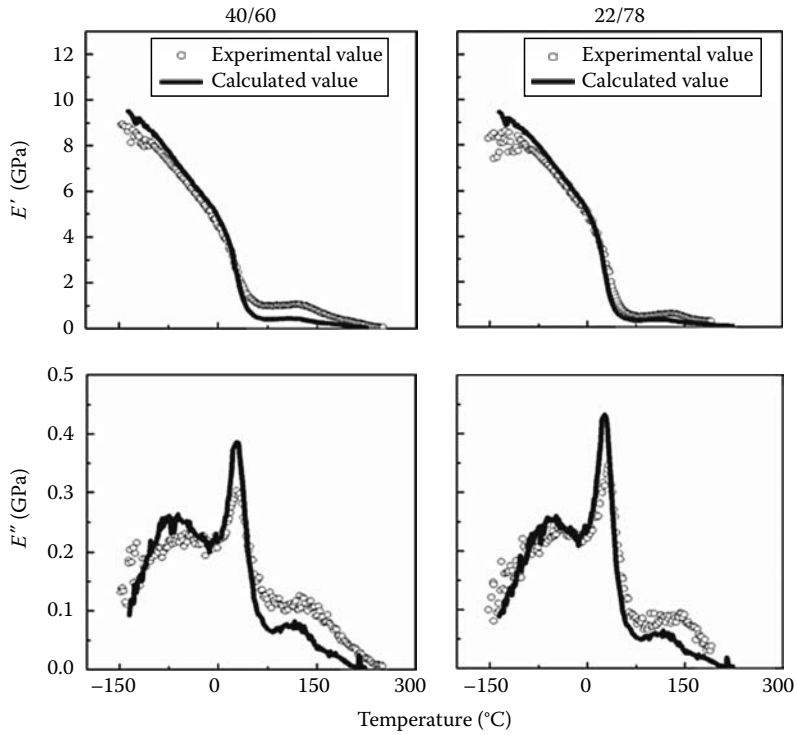

FIGURE 9.8 The theoretical and experimental results of the storage modulus (E') and the loss modulus (E'') at a frequency of 10 Hz for chitosan/PVA blend films (40/60 and 22/78). (From Nakano, Y. et al., *Macromol. Symp.*, 258, 63, 2007. With permission.)

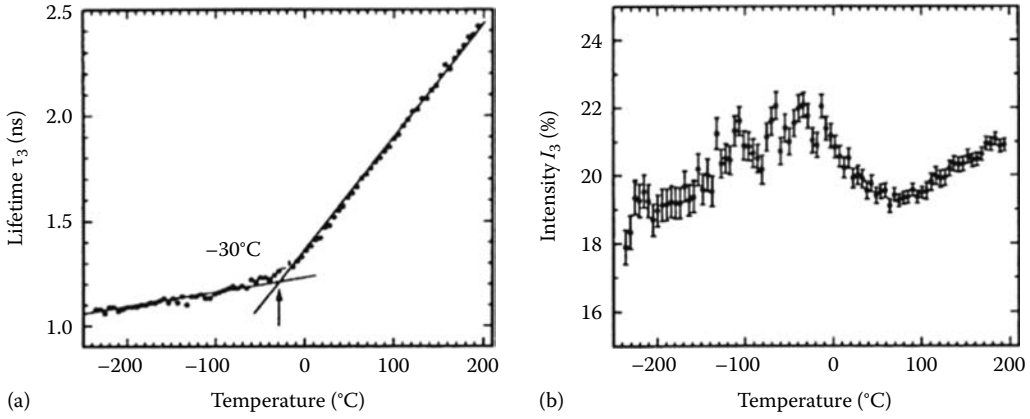

FIGURE 9.9 Temperature dependence of the corresponding (a) lifetime (τ_3) and (b) the intensity (I_3) for the long-lived component due to *o*-Ps pick-off annihilation of PVA film (0/100).

Figure 9.9a and b shows temperature dependence of the intensity (I_3) and the lifetime (τ_3), respectively, of the long-lived component due to *o*-Ps pick-off annihilation (Tao 1972, Cerna et al. 1989, Levey et al. 1989, Zhang et al. 1991, Suzuki et al. 1995) are given as a function of the temperature for the PVA film. The heating rate was 5°C/h. I_3 is the quantity of the *o*-Ps formation probability. Although τ_3 has been successfully correlated to the average size of the free volume holes presented in polymers, I_3 has been found to be influenced by many factors, such as the temperature, positron irradiation, electric field and polar group.

With increasing temperature, τ_3 increases as shown in Figure 9.9a. In this figure, there is one transition at ca. –30°C, which appeared at the same temperature as that for the γ dispersion peak, as shown in Figure 9.6. According to the correlation between τ_3 and the free volume size (Tao 1972), the γ-dispersion has been explained as being the contribution from the glass transition associated with the free volume due to localized motion of the local group of amorphous chains. If this is the case, the glass transition temperature (T_g) of PVA must be –35°C corresponding to the γ dispersion.

The T_g of PVA was reported to be around 85°C by Masci et al. (2003), on the basis of the appearance of the very broad peak, and in agreement with the value reported elsewhere (Sakurada 1985). On the other hand, Yang et al. (2004) estimated that T_g is ca. 71°C corresponding to a sudden change of DSC curve. Although 70°C–85°C is certainly the well-established value of T_g, the first transition of τ_3 suggests that T_g of PVA is around –30°C, which is in good agreement of a decrease of E' with temperature. If the glass transition temperature must be defined as the starting point of the drastic decrease in Young's/shear modulus, it must reflect the γ dispersion. Actually, it was confirmed that the first transition by positron annihilation corresponded to the γ dispersion for several kinds of polyethylene films and the glass transition was related to the γ dispersion (Matsuo et al. 2002, 2003). Unfortunately, the glass transition temperature of the chitosan film could not be discussed on the basis of the first transition of τ_3, since the values of τ_3 and I_3 were scattered.

As can be seen in Figure 9.9b, the temperature dependence of I_3 for PVA is similar to that of polyethylene (Matsuo et al. 2002). It can be seen that I_3 increases as a function of temperature up to –10°C. The increase in I_3 has been proved to be due to the positron irradiation effect on a polymer at low temperature. The secondary electrons that escape from the positron spur could be easily trapped in shallow potentials formed between the polymer chains when the motions of the molecular chains and groups are frozen at low temperature. Due to the positron irradiation time (experimental time), the probability of P_s formation would become larger. As can be seen in this figure, I_3 becomes a maximum at around –10°C, and begins to decrease with increasing temperature. I_3 attains a minimum at ca. 75°C and increases again beyond ca. 75°C. This is due to an apparent increase in the number of holes detected by positron annihilation, because of the thermal expansion of the holes at

elevated temperature. The very small holes, which could not be detected by positron annihilation at temperatures <75°C, can be detected by an increase in their size as a result of thermal expansion.

In accordance with Suzuki et al. (2000), the minimum temperature of I_3 is due to the fact that the local motion causes a large (macro-Brownian) correlated movement of the polymer chains in the amorphous phase. Accordingly, the movement may erase most of the shallow potentials; the trapped electrons may then also disappear. If this is the case, the molecular motion at low temperatures affects the concentration of trapped electrons in a shallow potential, and consequently, the variation in I_3 is closely related to the relaxation temperature as a second effect. In this experiment, it turns out that the β dispersion associated with the large movement of amorphous chains, approximately corresponds to the minimum region (75°C) of I_3, although the dispersion peak of E'' appeared around 30°C (see Figure 9.6). Interestingly, 75°C corresponding to the T_g (70°C–85°C) value of PVA reported elsewhere (Masci et al. 2003, Yang et al. 2004). This indicates that the established value of T_g is related to the local motion of large (macro-Brownian) movement. However, further consideration must be taken into account to lead to more conclusive evidence.

9.3.4 Surface Properties of the Blends

To study the surface characteristics, the peak separation attributed to the carbon atoms as confirmed by ESCA measurements was carried out for chitosan (100/0), chitosan/PVA blend (40/60 and 22/78) and PVA (0/100) films, the results of which are shown in Figure 9.10. Of course, the correction of the photoionization cross section was done. The peak separation was performed by the established method reported for C–C and C–H (peak 1), C–O (peak 2), C–NH (peak 3) and O–C=O (peak 4) (Anderson et al. 2001, Sigurdson et al. 2002, Ma et al. 2002, Sreedhar et al. 2006). The total curve (dotted curve) corresponding to the summation of each peak magnitude gave the best fit with respect to the measured curve (solid curve). The peak area fractions of the specimens are listed in Table 9.4. Peak 3 associated with chitosan became more intense with increasing draw ratio. This reveals that the chitosan content on the surface of the blend films increases with increasing draw ratio.

Figure 9.11 shows the N_{1s}/C_{1s} ratio with respect to the chitosan content for the undrawn blend films, and the draw ratio for chitosan (100/0) and chitosan/PVA blend (40/60 and 22/78) films, in which the N_{1s}/C_{1s} ratio corresponds to the normalized value of the N_{1s} peak area against C_{1s} peak area given as a summation of four components in Figure 9.10. As shown in Figure 9.11a, the component of the N_{1s} peak area associated with the content of chitosan is much smaller than that of the C_{1s} peak area. However, as shown in Figure 9.11b, the increasing tendency of the N_{1s}/C_{1s} ratio with respect to the draw ratio can be recognized. If the dispersion of chitosan is uniform within the blend film, the value must be independent of draw ratio. The increasing tendency is significant for the 40/60 blend, indicating that the chitosan content on the blend film surface increases with increasing draw ratio. This is probably thought to be due to the fact that the surface energy of chitosan is lower than that of PVA.

Figure 9.12a and b show the corresponding changes in contact angle with respect to the chitosan content and the draw ratio, respectively, for the indicated blend films. The wettability of the chitosan film is confirmed to be less than that of PVA, and then the contact angle increases with increasing chitosan content. The contact angle for the PVA film decreases with increasing draw ratio, since the fraction of C–O increases, as listed in Table 9.4. The decrease in contact angle becomes less pronounced with increasing chitosan content at each draw ratio. This phenomenon is obviously reasonable, since the ESCA results revealed decreases in the number of C–O and O–C=O groups on the surface. The increase in chitosan content shown in Figures 9.10 and 9.11 together indicates that the surface of the drawn blend film must be compounded with amorphous chitosan and PVA chains. Such a sufficient number of entanglement meshes between the amorphous chitosan and PVA chains relates to good miscibility, and is very important to ensure uniform elongation of the blend films. Actually, no macrocracking was found to occur under the elongation process, and the drawn blend film was transparent.

The similar tendency was confirmed for the blends of ultrahigh molecular weight polyethylene (UHMWPE) and ultrahigh molecular weight polypropylene (UHMWPP) prepared by

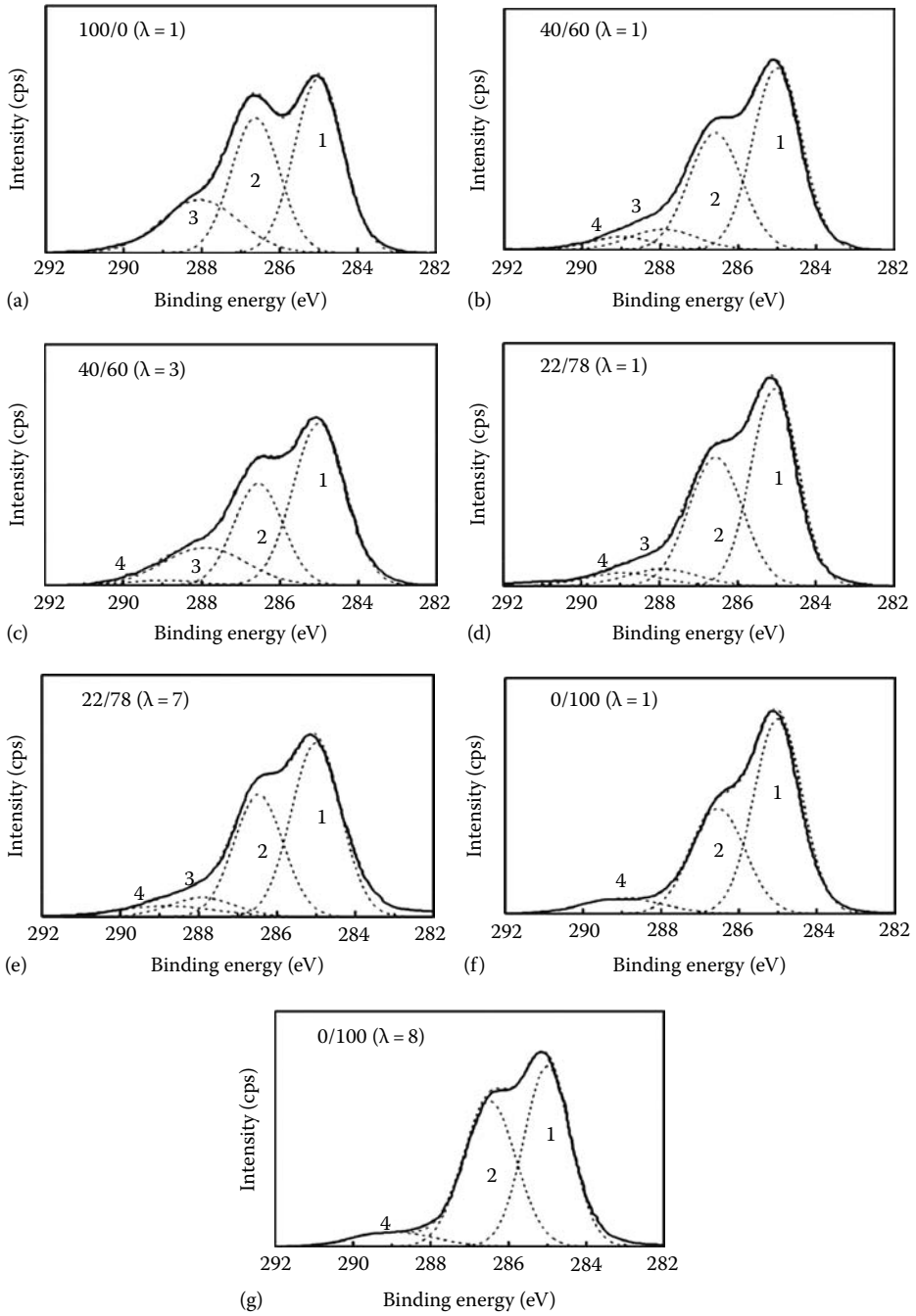

FIGURE 9.10 C_{1s} spectra afforded by ESCA measurements of (a) the undrawn chitosan film (100/0), (b)–(e) the undrawn and drawn chitosan/PVA blend films (40/60 and 22/78) and (f), (g) the undrawn and drawn PVA films (0/100). (From Nakano, Y. et al., *Macromol. Symp.*, 258, 63, 2007. With permission.)

gelation/crystallization method (Sawatari et al. 1987, 1990). The blend films provided intrinsic diffractions from the crystal planes of UHMWPE and UHMWPP indicating the separate crystallization of each chain. But the blend films could be elongated more than 80 times. Such high elongation is thought to be due to the entanglement meshes of UHMWPE and UHMWPP amorphous chains.

TABLE 9.4
The Peak Area of C_{1s} Components by ESCA Measurements

		Peak Area Ratio (%)							
Peak No.		**1 (C—C, C—H)**		**2 (C—O)**		**3 (C—NH)**		**4 (O—C=O)**	
Chitosan	(λ = 1)	44.38		34.11		21.51		—	
40/60	(λ = 1)	51.64		35.80		7.92		4.64	
	(λ = 3)		50.65		29.55		17.87		1.93
22/78	(λ = 1)	54.27		34.25		6.38		5.10	
	(λ = 7)		51.25		35.62		7.22		5.90
PVA	(λ = 1)	57.77		34.95				7.28	
	(λ = 8)		49.06		44.43	—	—		6.51

Source: Nakano, Y. et al., *Macromol. Symp.*, 258, 63, 2007. With permission.

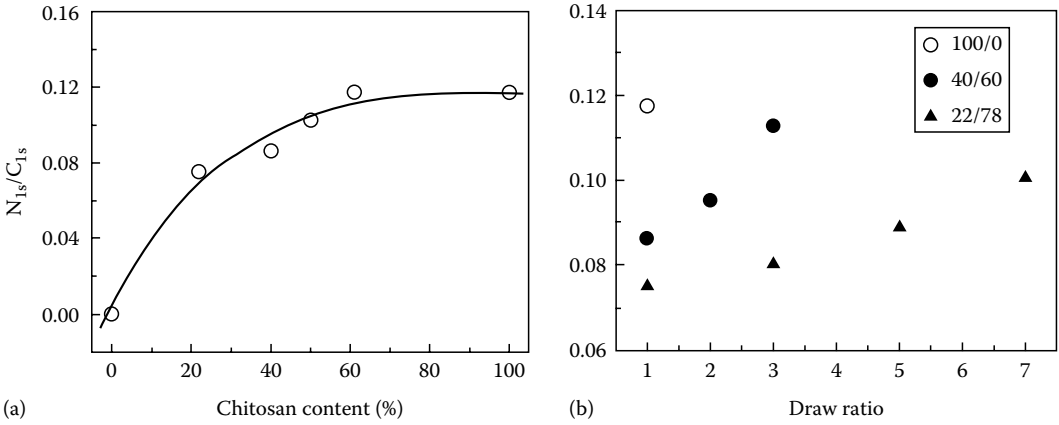

FIGURE 9.11 The N_{1s}/C_{1s} ratio afforded by ESCA measurements with respect to (a) chitosan content for the undrawn films and (b) draw ratio for chitosan/PVA blend films (40/60 and 22/78) in addition to undrawn chitosan film (100/0). (From Nakano, Y. et al., *Macromol. Symp.*, 258, 63, 2007. With permission.)

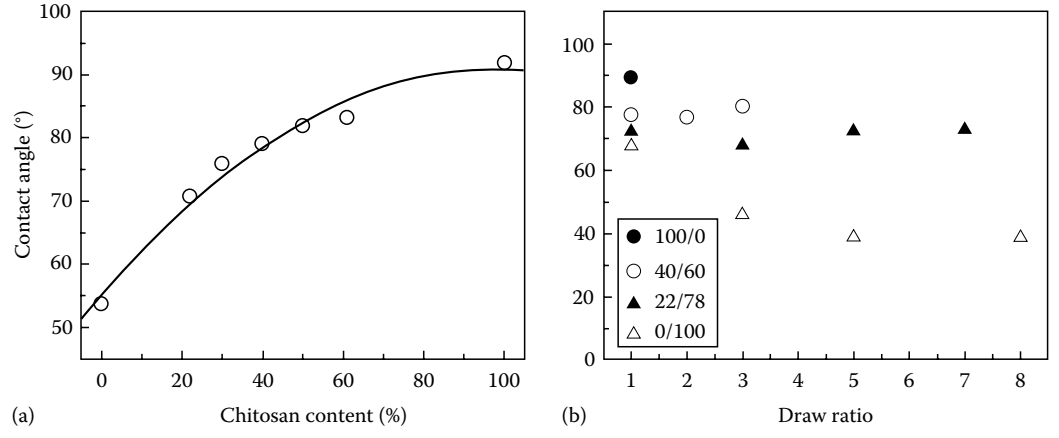

FIGURE 9.12 The change in contact angle with respect to (a) the chitosan content for the undrawn films and (b) the draw ratio for the blend films (40/60 and 22/78). (From Nakano, Y. et al., *Macromol. Symp.*, 258, 63, 2007. With permission.)

9.4 CONCLUSION

The morphology and physical properties of chitosan, chitosan/PVA blend and PVA films prepared from aqueous solution were investigated. The origins of the thermal and mechanical properties of chitosan and PVA with inter- and intrahydrogen bonds were investigated systematically by using positron annihilation, DSC, and x-ray and viscoelastic measurements. The broad endothermic peak appeared at 110°C–130°C by the first heating up to 160°C was associated with the evaporation of water containing the films and was hardly affected by the mobility of the amorphous chain segments of chitosan and PVA, since the E' and E'' curves for the first heating almost overlapped with the curves for the second heating run. The transition of τ_3 by positron annihilation for PVA was related to the micro-Brawnian motion, while the decrease of I_3 is related to macro-Brawnian motion. Namely, the transition of τ_3 and the decrease of I_3 were in good relationship with γ and β mechanical relaxations of the amorphous phase, respectively. The mechanical properties of the blend films were also analyzed as a function of chitosan content on the basis of the deviation from a simple additive rule of complex dynamic moduli between chitosan and PVA content ratio by a three-dimensional model. In this model system, a number of small chitosan fragments as islands are buried in an isotropic PVA phase as ocean. The calculated and experimental results indicated that the boundary between chitosan fragments and PVA matrix are connected strongly by their entanglements. Actually, the endothermic peak of the DSC curve resulting from admixing with chitosan slightly shifted to higher temperature, indicating co-crystallization leading to good miscibility. A detailed analysis afforded by contact angle and ESCA studies indicated that the chitosan content on the blend polymer surface increased with increasing draw ratio. Accordingly, it may be expected that the entanglement meshes between the chitosan and PVA amorphous chains on the surface assured uniform elongation without any macrocracking.

9.A APPENDIX

In the model in Figure 9.7, the chitosan layers are adjacent to the PVA layers with the interfaces perpendicular to the X_1, X_2, and X_3 axes fixed within the bulk specimen. The strains of the two phases at the boundary are assumed to be identical. The model is constructed from three composites, as shown in Figure 9.13. In model (a), the PVA layer (A) with fraction length $1 - \delta$ lies adjacent to the chitosan layer (C) with the interface perpendicular to the X_3 axis, and in model (b) the PVA layer with fraction length $1 - \upsilon$ is attached to the structure of phase I in a plane normal to X_1 the direction. The final phase III can be constructed by adding a $1 - \mu$ PVA layer with fraction length to phase II. Accordingly, the complex moduli E_I^* of phase I is given from the viewpoint of a series model.

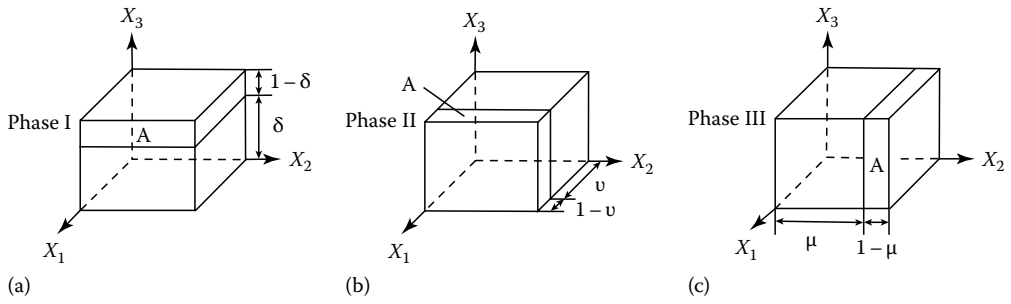

FIGURE 9.13 A composite procedure for the construction of a model in Figure 9.7. (a) PVA phase attached to the X_3 face of chitosan phase to construct phase I. (b) PVA phase attached to the X_1 face of phase I to construct phase II. (c) PVA phase attached to the X_2 face of phase II to construct phase III. (From Matsuo, M. et al., *Macromolecules*, 23, 3266, 1990. With permission.)

$$E_{\mathrm{I}}^{*} = \frac{\delta}{E_{1}^{*}} + \frac{1-\delta}{E_{2}^{*}} \tag{9.A.1}$$

By using a concept of the parallel model, the modulus E_{II}^{*} of phase II and $E_{\mathrm{III}}^{*}(E^{*})$ of phase III may be given, respectively, as:

$$E_{\mathrm{II}}^{*} = \upsilon E_{\mathrm{I}}^{*} + (1-\upsilon)E_{2}^{*} \tag{9.A.2}$$

and

$$E_{\mathrm{III}}^{*} = \upsilon E_{\mathrm{II}}^{*} + (1-\mu)E_{2}^{*} = E^{*} \tag{9.A.3}$$

Substituting Equations 9.A.1 and 9.A.2 into (9.A.3), Equation 9.1 may be obtained.

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10 Electrostatic Properties of Chitosan

Won Jong Kim

CONTENTS

10.1 INTRODUCTION

Chitosan is a natural polysaccharide possessing biocompatible and biodegradable (Muzzarelli 1997, Muzzarelli and Muzzarelli 2002) properties. It has received great attention from researchers due to its wide application in medical and pharmaceutical fields. Its favorable intrinsic properties and highly abundant natural resources make it a versatile utility material for various medical applications such as topical ocular application (Felt et al. 1999), implantation (Patashnk et al. 1997) or injection (Song et al. 2001). Owing to its positive charges at physiological pH, chitosan could bind itself to the negatively charged surfaces of most of the cells and living tissues and thus also used as bioadhesive, which increases retention of the therapeutic material at the site of application (Calvo et al. 1997, He et al. 1998). Chitosan is used as a component in hydrogels and has been exploited in various medical and pharmaceutical applications especially in sustained release of anticancer drugs, delivery of sensitive biological materials such as proteins and living cells, and release of macromolecules over a period of several hours to a few days in a controlled manner.

Chitosan is a copolymer of β-[1–4]-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit) (Figure 10.1), and is generated from chitin, which is the main component of the exoskeleton of crustaceans, such as shrimps (Muzzarelli 1973). Deacetylation of chitin under alkaline condition produces chitosan. The main parameters that influence the characteristics of chitosan are its molecular weight (MW) and degree of deacetylation (DD), representing the proportion of deacetylated units. These parameters can be controlled by selecting appropriate conditions during preparation and can be further modified at a later stage by altering DD through reacetylation (Sorlier et al. 2001) or deacetylation as per requirements or by lowering the MW through acidic depolymerization (Dong et al. 2001).

Unlike other naturally occurring polysaccharides, e.g., cellulose, dextran, pectin, alginic acid, agar, agarose and carrageenans, which are neutral or acidic in nature, chitosan is a highly basic polysaccharide. This unique property imparts several functional attributes such as polyoxysalt formation, ability to form films, chelation with metal ions, and optical structural characteristics (Hench 1998).

Irrespective of the chemical compositions, both the acetylated and deacetylated units in chitosans exist in the $^4C_1$ ring conformation, which implies complete diequatorial glycosidic linkages. This

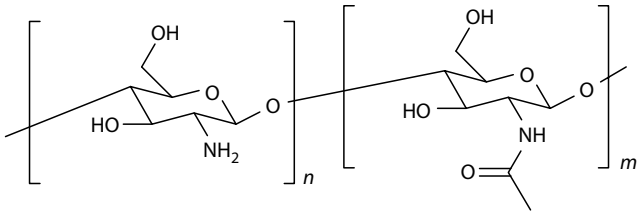

FIGURE 10.1 Chitosan.

diequatorial geometry of the glycosidic linkage restricts rotation in linked polymers, which results in rigidity of the structure that eventually leads to highly extended chains and high intrinsic viscosity in solution (Anthonsen et al. 1993). Highly crystalline structure of chitosan arising from extensive interchain hydrogen bonding is evident from the poor solubility of chitosan under physiological conditions and in organic solvents. However, chitosans with low degree of deacetylation (<40%) are soluble up to pH 9, whereas highly acetylated chitosans (>85%) are soluble only up to pH 6.5.

Chitosan is a weak base due to the presence of D-glucosamine residue and has a pK_a value of 6.2 and therefore is insoluble at neutral and alkaline pH values. However, it forms salts with inorganic and organic acids such as hydrochloric acid, acetic acid, glutamic acid, and lactic acid. Chitosan molecules have a strong positive charge at pH < 6.2, which allows them to bind with negatively charged molecules or polyanions through strong electrostatic interactions. Addition of salts to the solution increases the ionic strength and lowers the solubility of chitosans due to the charge neutralization by increased counterion concentrations.

10.2 POLYELECTROLYTE PROPERTIES

In general, the polymer complexes could be categorized as hydrogen-bonding complexes, stereocomplexes and charge-transfer complexes, and polyelectrolyte complexes (PEC) (Que and Jiang 1995) depending upon the attractive forces responsible for binding the components together. In particular, the PECs are generated by the electrostatic interaction of oppositely charged polymers. As PEC requires two oppositely charged macromolecules, both the polymers must be ionized and possess opposite charges. Therefore effective PEC formation can only take place at a certain pH range that is within the pK_a values of the two polymers. Generally the charge ratio of anionic-to-cationic polymers, the degree of neutralization, the ionic strength as well as the valence of simple ions present in the electrolyte solution, play important roles in the formation and regulating physicochemical properties of polymer complexes (Kekkonen et al. 2001, Gamzazade and Nasibov 2002). Anions and cations from strong acids and strong bases, respectively, usually give rise to a strong PEC. The polymers, which undergo complete ionization under experimental conditions, can also form strong polyelectrolyte complexes. However, weak acids and bases provide weak polyelectrolyte complexes (Denuziere et al. 1996).

The prevalent use of PECs can be demonstrated in various applications, such as membranes, medical prosthetics, antistatic coatings, environmental signals to sensors, drug and gene delivery systems, and protein separation systems, etc. (Arguelles-Monal et al. 2000). Natural polymers, such as polysaccharides, which are endowed with additional favorable attributes like nontoxicity and bioabsorbability (Cascone et al. 1995), are regarded as very potent PECs. The presence of amino groups in the glucosamine residue imparts an additional characteristic feature to chitosan in comparison to these polysaccharides. The positive charge arising due to the highly protonated amino functionalities enables chitosan to form PEC spontaneously with a wide variety of negatively charged polyanions such as lipids, collagen, glycosaminoglycans, lignosulfonate, and alginate, as well as charged synthetic polymers and DNA through electrostatic interaction. These complexes are generally water insoluble and are known to form hydrogels. Variation in chemical structure of component polymers gives rise to diversities in the physiochemical properties of the polyelectrolytes. The variable parameters such as molecular weight, flexibility, functional group structure, charge

density, hydrophilicity and hydrophobicity, stereoregularity, and compatibility, as well as reaction conditions like pH, ionic strength, polymer concentration, mixing ratio, and temperature could be controlled to achieve much desired diversity of physical and chemical properties of the complexes.

The chitosan-based polyelectrolytes have wide applications in drug delivery, nonviral vector-mediated genes delivery (Erbacher et al. 1998, Borchard 2001, Thanou et al. 2002, Okamoto et al. 2003, Nagasaki et al. 2004) and in many uses as in biospecific sorbents (Sashiwa et al. 2000, Sharma et al. 2003), films (Shu et al. 2001, Sasaki et al. 2003, Park and Zhao 2004), and gels (Ruel-Gariepy et al. 2000, Ramanathan and Block 2001, Vinogradov et al. 2002, Kofuji et al. 2004).

10.3 ELECTROSTATIC INTERACTION OF CHITOSAN WITH DNA

As chitosan bears amino functionalities in its glucosamine residues, it can form cationic polymers. The charge of the polymer depends upon the degree of protonation of these amino groups. Mumper et al. (1995) was the first to demonstrate the effective polyelectrolyte formation between negatively charged DNA and chitosan. The report triggered extensive studies dealing with the chitosan/DNA complexation due to the immense potential of these polyplexes as nontoxic, biodegradable, and nonviral vector for gene delivery. There were several published reports (Illum et al. 1994, Aspden et al. 1997) relating to the complexation between chitosan and DNA with special references to their morphology, particle size, electrostatic charge of the resulting polyplexes, and compaction and these key physicochemical features were known to play important role in effective gene delivery.

The strong complexation of chitosan and DNA arises due to the strong electrostatic interaction between the positively charged protonated chitosan and the negatively charged DNA and ensures the entry of the intact polyplexes into the cell.

The extent of DNA complexation or encapsulation into chitosan nanoparticles is determined by various methods. One of the most common means to evaluate the extent of effective complexation is the competitive binding studies by agarose gel electrophoresis using ethidium bromide (EtBr) as DNA stain. EtBr fluoresces under UV light when intercalated into DNA (or RNA). DNA migrates through an EtBr-treated gel under the applied electric field and it becomes distinctly visible under UV light as a band. Upon addition of chitosan to a solution of EtBr-stained DNA, fluorescence decreases as a result of competitive binding of cationic chitosan to DNA. A combination of EtBr staining and confocal laser scanning microscopy has also been reported to determine the distribution of DNA in chitosan nanoparticles. Another alternative method for determining DNA loading is the Pico Green assay performed after digestion with chitosanase and lysozyme (Leong et al. 1998).

The method of complex formation also regulates the size, morphology, and zeta potential of the complexes. A simple mixing (Mumper et al. 1995) of chitosan with DNA gives rise to broad particulate complexes having mean size between 100 and 600 nm, which is in turn dependent on the molecular weight of chitosan. The zeta potential values vary between +0 and 20 mV with the extent of molar excess of chitosan (Cui and Mumper 2001). In other approach (Mao et al. 2001), the method for nanoparticle formation was modified by increasing the mixing speed and temperature during the blending of Na_2SO_4-containing DNA solution and the polymer solution. These modifications resulted in significant change in morphology of the monodispersed, spherical nanoparticles having particle size in the range of 200–500 nm. Though it was established that neither sodium sulfate concentration (2.5–25 mM) nor plasmid size exerted any effect on the particle size, the molecular weight of chitosan, and the N/P ratio played a significant role in determining the particle size (Thanou et al. 2002, Janes and Alonso 2003).

These complexes, known as chitosan/DNA nanosphere, differ from the traditional complexes due to their compact structure and displayed novel properties. The molecular weight and DD of chitosan is the crucial parameter that controls DNA compaction, the nuclease resistance of the complexes, and the dissociation of DNA from chitosan (Köping-Höggård et al. 2001, Sato et al. 2001). It was reported that the zeta potential, cellular uptake, and cytotoxicity of DNA/chitosan were dependent on DD value of chitosan but independent of the molecular weight of chitosan (Huang et al. 2004).

10.4 LOW-MOLECULAR-WEIGHT CHITOSAN AS A pH-RESPONSIVE ACTUATOR OF DNA REACTION

The complexation ability of low-molecular-weight water-soluble chitosan (LWMWC) and its pH responsiveness have been judiciously utilized in designing a novel proton-driven DNA strand rearrangement actuator based on chitosan (Lee et al. 2009). The operation of DNA nanomachine relies on the process of DNA strand rearrangement or exchange. This process is solely controlled by stabilization of DNA molecules through associative and dissociative forces arising from base pair interaction (Figure 10.2). Briefly, the initial double-stranded DNA (dsDNA) forms three-stranded DNA intermediate through partial association with complementary single-stranded DNA (ssDNA). This intermediate on subsequent dissociation through branch migration gives rise to more stable dsDNA and ssDNA. Thus, highly effective DNA reaction actuator is expected to make DNA nanomachine more flexible, controllable, and powerful device.

The DNA nanomachines reported so far are mainly based on the reversible process of protonation and deprotonation of DNA itself apart from other stimuli-responsive processes. However, these DNA-protonation-based nanomachines are restricted to specific DNA sequences. Therefore to develop improved DNA nanomachine having greater flexibility and superior controllability Kim's group (Lee et al. 2009) devised new strategy using chitosan, which could control the DNA strand rearrangement and provide wider applicability. Their earlier work demonstrated that the cationic comb-type copolymer, PLL-*g*-Dex, accelerates the DNA strand exchange reaction between dsDNA and complementary ssDNA (Kim et al. 2001, 2003, Yamana et al. 2005). Furthermore, those cationic copolymers considerably increased the responsiveness and robustness of DNA nanomachines (Choi et al. 2007) and could interact with negatively charged DNA and help the machine to move quickly and efficiently without careful adjustment of micro-conditions. However, PLL-*g*-Dex copolymer is unresponsive to exogenous stimulus such as pH. Thus, incorporation of a stimuli-responsive substance such as a pH-responsive polymer in DNA strand rearrangement could make this machine more efficient, flexible, and tunable. Polycations having high pK_a value interact with DNA too strongly and do not have pH-responsive complexation ability with DNA, so they are not suitable for the purpose. High-molecular-weight chitosan (HMC) has excellent interaction with both high-molecular-weight plasmid DNA and low-molecular-weight oligodeoxynucleotide (ODN, ~20 bp), and its ability to form complex with ODN is independent of the molecular weight of nucleic acids (Lee et al. 2001, Nah and Jang 2002). However, the poor solubility of HMC under physiological conditions and in organic solvents due to its highly crystalline structure limits its potential as an actuator. In contrast, chitosan having low molecular weight becomes protonated and deprotonated depending upon the pH of the media. Moreover, LMWSC exhibits higher solubility and much lower immunogenicity in spite of its poor polyelectrolyte complex–forming ability with ODN in comparison to HMC under physiological conditions. Though LMWSC interacts poorly with ODN at neutral pH condition, it may form a polyelectrolyte complex with ODN in acidic conditions. The effect of LMWSC on DNA strand rearrangement reaction at different pH conditions was evaluated by both gel electrophoresis and fluorescence resonance energy transfer (FRET) assay with high resolution.

At first, the pH-responsive complexation of LMWSC to ODN was investigated by a gel retardation assay (as shown in Figure 10.3a,b). The negatively charged DNA moves to anode under the

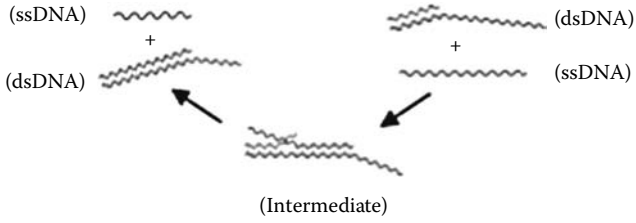

FIGURE 10.2 DNA strand rearrangement. (Lee, D., et al., *Mol. BioSyst.*, 5, 391, 2009. Reproduced by permission of The Royal Society of Chemistry.)

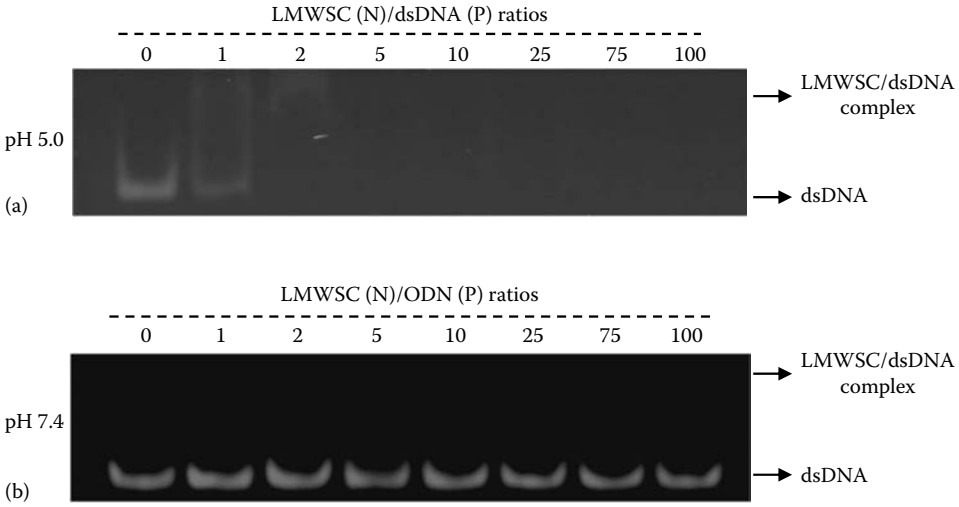

FIGURE 10.3 Complexation profile between LMWSC and DNA with various N/P ratios at indicated pH. LMWSC/DNA complexes with various N/P ratios (ranging from 0 to 100) were prepared by incubating mixtures of LMWSC and dsDNA in phosphate-buffered saline (PBS, pH 5.0 and pH 7.4) at 37°C for 1 h. Then, the complexes were electrophoresed on 13% polyacrylamide gel for 1 h at 100 V. (Reproduced by permission of The Royal Society of Chemistry.) (Lee, D., et al., *Mol. BioSyst.*, 5, 391, 2009. Reproduced by permission of The Royal Society of Chemistry.)

applied electric field during gel electrophoresis experiment. On formation of ionic complex between DNA and LMWSC, the DNA band, however, is expected to be retarded and no movement takes place. As expected, no band corresponding to dsDNA was observed at N/P (LMWSC nitrogen/ODN phosphate) ratios over 2 under UV illumination when the gel retardation assay was carried out at acidic condition (pH 5.0) (Figure 10.3a). However, at neutral pH of 7.4 even at a higher N/P ratio of 100 (Figure 10.3b), no complexation was observed.

After establishing the unique pH-responsive property of LMWSC in complexing DNA, they evaluated the LMWSC-mediated DNA strand rearrangement study at different pH conditions as shown in Figure 10.4. The fluorescence-labeled double-stranded DNA (F-dsDNA) was incubated with complementary nonlabeled ssDNA so that any displacement of F-dsDNA by complementary nonlabeled ssDNA would result in appearance of lower band corresponding to F-ssDNA. At physiological pH of 7.4 (Figure 10.5a), no displacement of F-dsDNA by complementary nonlabeled

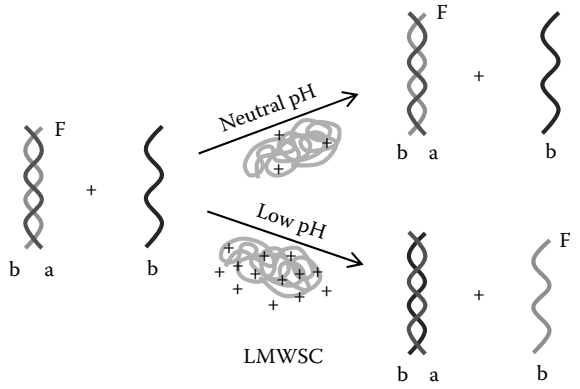

FIGURE 10.4 Schematic representation of pH-dependent LMWSC-mediated DNA strand rearrangement reaction. (Reproduced by permission of The Royal Society of Chemistry.) (Lee, D., et al., *Mol. BioSyst.*, 5, 391, 2009. Reproduced by permission of The Royal Society of Chemistry.)

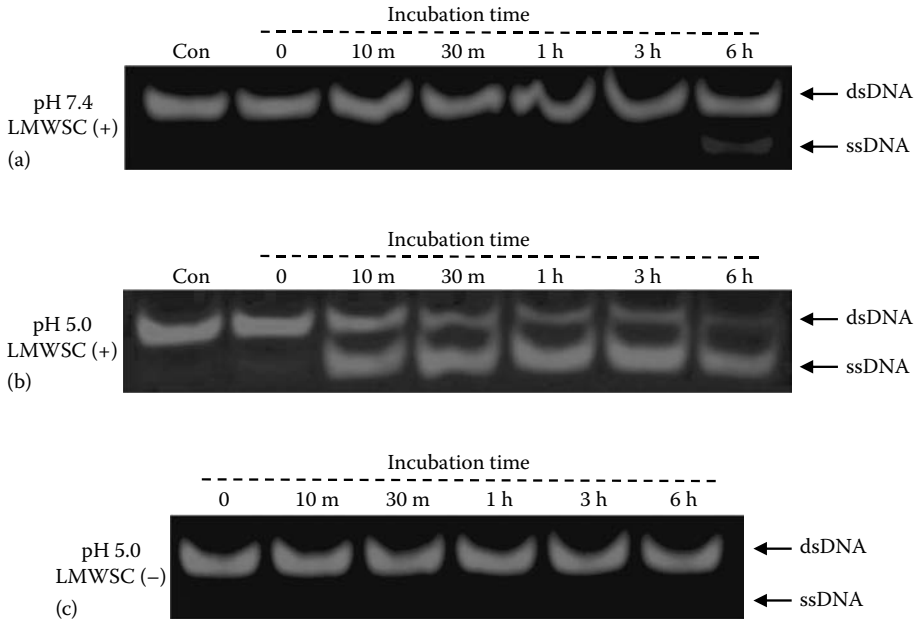

FIGURE 10.5 DNA strand exchange reaction at pH 7.4 (a) and at pH 5.0 (b) in presence of LMWSC. (c) DNA strand exchange reaction at pH 5.0 without LMWSC. Before gel electrophoresis, 1.5% SDS was added to the reaction mixture to dissociate both of dsDNA and ssDNA from LMWSC for visualizing the fluorescence images of DNA bands. (Reproduced by permission of The Royal Society of Chemistry.) (Lee, D., et al., *Mol. BioSyst*., 5, 391, 2009. Reproduced by permission of The Royal Society of Chemistry.)

ssDNA took place even in the presence of LMWSC as evident from the absence of a lower band corresponding to F-ssDNA. However, at pH 5.0, the DNA strand exchange reaction was accelerated to begin within 10 min and was almost complete within 30 min (as shown in Figure 10.5b.). The experiment was repeated at pH 5.0 in absence of LMWSC, but no DNA strand exchange has been observed. The observations clearly indicated the indispensability of LMWSC in regulating the pH-mediated DNA strand exchange reaction. Further investigation revealed that the extent of DNA strand exchange was dependent on the amount of LMWSC. At lower N/P ratio, the DNA strand exchange was not complete, however at N/P ratio 10, the degree of DNA strand exchange attained its maximum magnitude.

In order to estimate the time course of the strand exchange reaction with high resolution, FRET assay was performed. The dsDNA was labeled with both fluorescein isothiocyanate (FITC) and a black hole quencher (BHQ). In the dsDNA state, the fluorescence emission from FITC is quenched by the BHQ, therefore no fluorescence could be detected from unperturbed dsDNA. When the strand exchange reaction proceeds, the fluorescence emission is expected to be recovered by the detachment of FITC-ssDNA from the initial double-labeled dsDNA. In the subsequent experiments, the double-labeled dsDNA was then subjected to DNA strand exchange reaction with nonlabeled complementary ssDNA in presence and in absence of LMWSC at pH 5.0 and 7.4. As shown in Figure 10.6, the strand exchange reaction was accelerated dramatically only at pH 5.0 in presence of LMWSC, while no strand exchange reaction was observed in absence of LMWSC even at pH 5.0.

The plausible mechanism of DNA strand exchange (or rearrangement) reaction between dsDNA and complementary ssDNA could be explained with two distinct pathways, a dissociative pathway and a sequential displacement pathway (Broker and Lehman 1971, Reynaldo et al. 2000). The dissociative pathway reaction can be initiated by the spontaneous and complete dissociation of preformed dsDNA followed by the association of complementary ssDNA. However, the probability of

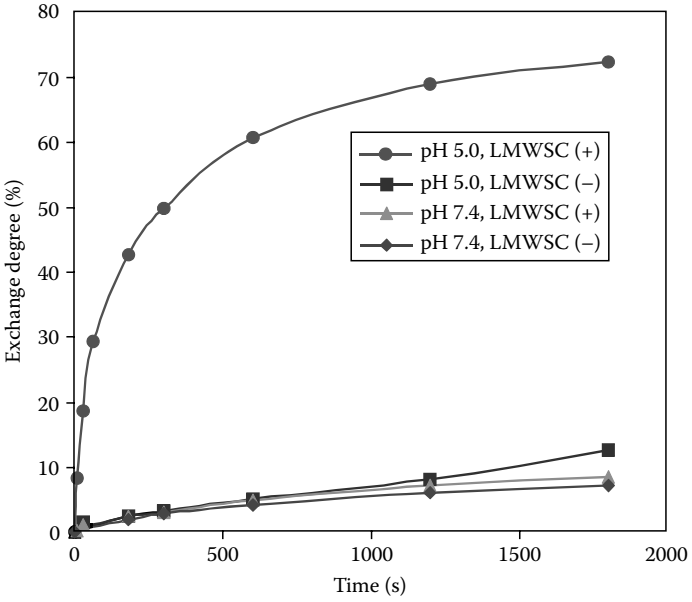

FIGURE 10.6 Time course of DNA strand exchange reaction. The doubly labeled dsDNA with FITC and BHQ1 was mixed with non-labeled ssDNA in presence as well as in absence of LMWSC at indicated pH. Exchange degree was calculated from observed fluorescence intensity. (Reproduced by permission of The Royal Society of Chemistry.) (Lee, D., et al., *Mol. BioSyst*., 5, 391, 2009. Reproduced by permission of The Royal Society of Chemistry.)

this path could be ruled out under physiological condition as the reaction requires destabilization of highly stable dsDNA, which can only be achieved either by increasing the temperature over the melting point or by adding a destabilizing reagent. On the other hand, the sequential displacement pathway requires only a partial dissociation of dsDNA, followed by branch migration and complete exchange reaction. Maruyama et al. reported the cationic comb-type copolymer-mediated strand exchange and its underlying mechanism (Kim et al. 2002). At pH 5.0, LMWSC acquired sufficient positive charges and thus stabilized the formation of a three-stranded intermediate whose formation was hindered by the entropically unfavored accumulation of counterions and electrostatic repulsion between DNAs.

The stabilization the reaction intermediate of the strand exchange reaction arose from the spreading out of the accumulated counterions and concomitant increase of the entropy within the system. However, at physiological pH, LMWSC did not seem to be protonated enough to form a complex with short chain ODN, and therefore failed to diminish the electrostatic repulsion among three-stranded intermediate in the strand exchange reaction. The stabilizing effect of LMWSC on the association of complementary DNA was confirmed by melting temperature (T_m) assay. The LMWSC increased the T_m over 19°C at pH 5, while there was no change in T_m at neutral pH. In brief, LMWSC worked as a DNA strand rearrangement actuator fueled by protons in reaction buffer and it could be a major operating system in a DNA nanomachine. Consequently, this intelligent proton-driven DNA reaction actuator has the potential for the precise control of a DNA nanomachine and would be a possible component for operating and controlling a nanoscaled machine.

10.5 CONCLUSIONS

In conclusion, it can be inferred that the intrinsic electrostatic properties of chitosan coupled with its favorable physiochemical properties could extend the utilization of chitosan in medical, biomedical, industrial, and many other applications.

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11 Applications of Mass Spectrometry to Analyze Structure and Bioactivity of Chitooligosaccharides

Martin G. Peter and Marcos N. Eberlin

CONTENTS

11.1 INTRODUCTION

Advanced soft ionization MS methods are nowadays frequently used for the analysis of chitooligosaccharides (CHOs). The advantages of applying this method are the requirement of minute quantities of sample, allowing rapid detection of nano- to picomol quantities of sample, and the possibility to separate the components of even complex mixtures by mass. Especially, tandem MS yields a wealth of information and is the method of choice for structure analysis of higher CHOs. Free sugars can be analyzed and the preparation of derivatives for the purpose of increasing volatility, such as, e.g., trimethlylsilyl ethers (Coduti and Bush 1977), is not required.

Another powerful method for structure analysis of CHOs is NMR spectroscopy. Larger amounts of sample are required and the information obtained is limited to identification of three neighboring monosaccharide units. For higher CHOs, NMR shows the relative abundance of diads and triads in the oligosaccharide chain (Vårum et al. 1991a,b), which is useful for understanding the physicochemical properties of polymers.

The principles of CHO structure are explained in this chapter and applications of MS for structure determination of CHOs obtained by chemical or enzymatic methods as well as applications of MS for investigations of the kinetics and mechanisms of chitinolytic enzymes are reviewed. We will not consider stereoisomerism due to α- and β-anomeric configurations, investigations on *N*-acetylhexosaminidases, studies on *N*-glycans of glycoproteins containing a *N*,*N*′-diacetylchitobiose core, detection of chitinous materials in biological samples by pyrolysis GC-MS, and use of MS for

structure confirmation of synthetic compounds. With a few exceptions, lipo-CHOs are not included here, despite their close structural relationship to CHOs and their fundamental biological significance (for a review on MS of lipo-CHOs, see Van der Drift et al. 1998). Further, we will not consider techniques that are not in use anymore, except when they are of historical interest, representing a milestone during their time.

11.2 PRINCIPLES OF CHO STRUCTURE AND NOMENCLATURE

In chemical terms, the structure of any compound is defined by the connectivity of all atoms and their stereochemistry, including configurations of elements of chirality and conformations of the molecule. Structure analysis of CHOs by MS is limited to the determination of the number of D and A residues and their location in the oligosaccharide chain, i.e., their sequence. Stereochemical information is usually not obtained by MS and α/β-anomers of CHOs must be analyzed by NMR spectroscopy or by chromatographic separation, the latter being ideally coupled with MS (Fukamizo et al. 1994, Suginta et al. 2005). Otherwise, stereoisomerism is not relevant, as CHOs are always composed of β-1,4-linked D and/or A monosaccharide units. When drawing formulae, the conformation of CHOs is conveniently represented in the low-energy $^4C_1$ chair and the glycosidic bonds are depicted in *g*,*g*-conformations (cf. Germer et al. 2003).

CHOs that are composed exclusively of D or of A units are named homo-CHOs whereas those containing both D and A units are called hetero-CHOs (Figure 11.1). They are represented here as D_yA_z, where *y* and *z* are the numbers of D and A residues, respectively. CHOs differing in degree of polymerization (DP) are named oligomers, irrespective of their F_A values (Figure 11.2). Thus, for example, a tetrasaccharide (DP 4) is a lower oligomer of a pentasaccharide (DP 5). Oligomers of the same DP but differing in F_A are homologs, e.g., pentasaccharide D_4A_1 (F_A 0.2) is a lower homolog of pentasaccharide D_2A_3 (F_A 0.6). Given a particular homolog, isomers may exist that differ in the sequence of D and A units. The number of possible isomers is obtained by permutation of the sequence positions of D and A residues and is calculated by Equation 11.1. Thus, a pentasaccharide D_2A_3 may occur as ten isomers. Two of them, DADAA and DAADA, are shown in Figure 11.2. The number of isomers increases not only with DP, but also with similar values of *y* and *z*, as illustrated with a few examples in Table 11.1.

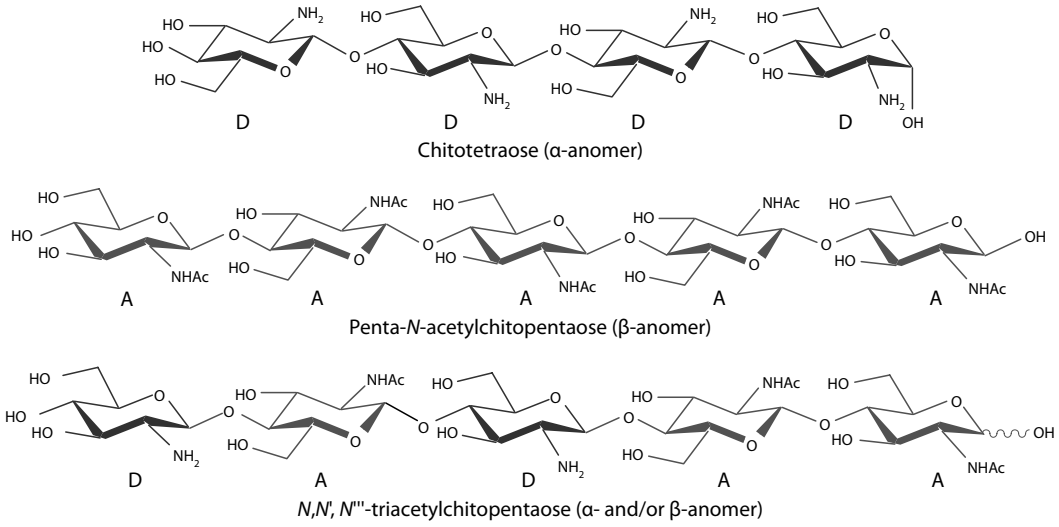


FIGURE 11.1 Examples of two homo-CHOs (chitotetraose and penta-*N*-acetylchitopentaose) and a hetero-CHO (*N*,*N*′,*N*‴-triacetylchitopentaose).

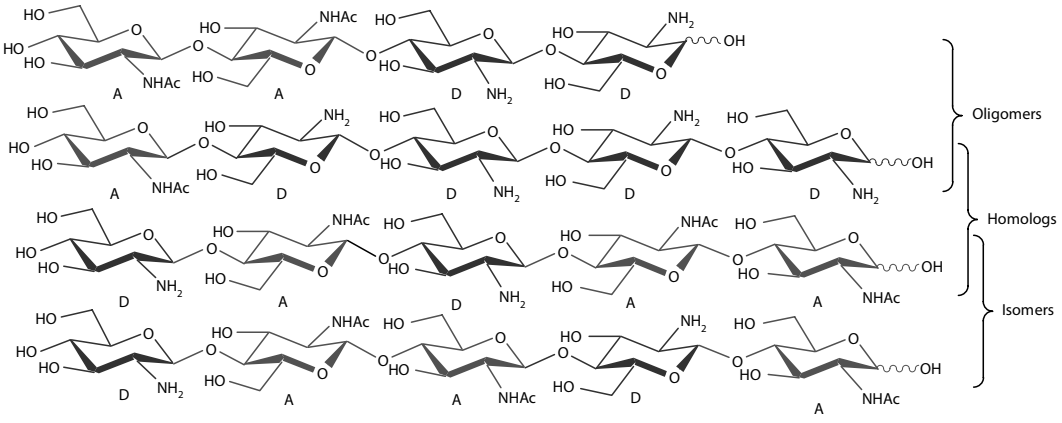


FIGURE 11.2 Examples of CHO oligomers, homologs, and isomers.

TABLE 11.1
Number of Possible Isomers of Some Oligomers and Homologs D_yA_z

DP	F_A	y	z	n
2	0.00	2	0	1
2	1.00	0	2	1
2	0.50	1	1	2
3	0.67	1	2	3
4	0.75	1	3	4
4	0.50	2	2	6
5	0.60	2	3	10
6	0.67	2	4	15
6	0.50	3	3	20
9	0.44	5	4	126
12	0.42	7	5	792

$$n = \frac{(y+z)!}{y! \times z!} \tag{11.1}$$

where
n = number of isomers
y = number of D residues
z = number of A residues = DP – y

11.3 PRINCIPLES OF STRUCTURE ANALYSIS OF CHOs BY MASS SPECTROMETRY

The chemical procedures and instrumental techniques used for analysis of CHOs are essentially the same as those developed in the field of glycobiology (for recent reviews, see Budnik et al. 2006, Fernandez 2007). A detailed description of the various instruments and ionization techniques is beyond the scope of this chapter, and the reader is referred to some textbooks (Dass 2000, 2007).

The determination of the molecular mass is the first step in MS analysis of CHOs. For MALDI-TOF MS, the sample is dissolved in water/methanol at a concentration of 0.1–1.0 mg/mL. An aliquot of 0.5 µL of the solution is mixed on the target with 2 µL of a 10%–15% solution of DHB, THAP, or CHCA in methanol (Mohr et al. 1995). After evaporation of the solvent and crystallization of the analyte/matrix mixture, the sample is irradiated with a pulsed laser beam, typically from a nitrogen laser ($\lambda = 337$ nm). The mass spectra of CHOs are nearly always recorded in the positive ion mode, though negative ion mass spectra may yield additional useful information. Positively charged ions are generated as the protonated molecules or adducts with a metal ion, i.e., Na^+ or K^+, the latter being present in trace amounts in the sample or the matrix or may be added by mixing a solution of a salt on target. Other cationic adducts may be generated by addition of different salts, e.g., LiCl (North et al. 1997). As the spectra may show more or less strong peaks of the matrix, it is essential to run blanks, besides using highly pure reagents.

In the other most widely applied method, i.e., ESI-MS, the sample solution is introduced into a metal capillary, the end of which bears an electrostatic potential, into the first vacuum stage of the mass spectrometer where it forms an aerosol. Evaporation of the solvent results in concentration of ionic species and finally in Coulomb explosion. Similarly to MALDI-MS, ions appear as protonated molecules or adducts with Na^+ or K^+. If desired, the sample solution may be spiked with a small amount of a salt or an acid, e.g., formic acid.

MALDI-MS gives little or no fragmentation and thus is the method of choice for molecular mass determination of components of complex mixtures. In contrast, doubly charged ions, e.g., $[M + 2H]^{2+}$, and well as fragmentation processes are more frequently observed in ESI-MS. Other MS soft ionization methods, such as CI or FAB, have been applied to CHO analysis but are currently rarely used.

The monoisotopic mass M of any CHO is calculated according to Equation 11.2:

$$M = (y \times 161.06881) + (z \times 203.07937) + 18.01056 \tag{11.2}$$

The theoretical masses of monocharged positive ions are calculated by adding 1.00783 (for $[M + H]^+$), 22.98977 (for $[M + Na]^+$), or 38.96371 (for $[M + K]^+$), respectively, to the mass value obtained according to Equation 11.2 (Figure 11.3). A note of caution may be appropriate: the deviation of the calculated from the experimental mass values should be <1 for m/z < ca. 1000 and <2 for m/z > ca. 1000. There are a few examples of wrong peak assignments in the literature. We have noted one publication (not cited here) where the experimental masses failed to match the theoretical mass of any CHO, and even two different m/z values were assigned to the same homolog.

It is sometimes said in the literature that the peak intensities observed in MALDI-MS are a measure for estimating the relative molar quantity of any CHO present in a mixture. When two components of a mixture would have the same ionization energy, peak intensity would be inversely correlated with molecular mass. However, according to our experience, the sensitivity of CHOs varies considerably with F_A (Figure 11.4). *N*-deuterioacetylation of hetero-CHOs by means of hexadeuterio-acetic anhydride introduces a $[^2H_3]$-acetyl group with a mass increment of 3 into every D residue. The resulting CHOs of the composition $([^2H_3]\text{-A})_y A_z$ are chemically indistinguishable from the corresponding homo-CHOs of the same DP, i.e., A_{y+z}. The correlation of peak intensity and relative molar amount is obviously valid in this case (Haebel et al. 2007).

The limit of detection of the DP of CHOs is unknown. With MALDI-MS, the record stands at ca. m/z 7000, corresponding to D_{43}, detected in a MMW fraction, which had been obtained after acid-catalyzed depolymerization of fully deacetylated chitosan ($F_A < 0.01$) (Figure 11.5) (cf. Bosso and Domard 1992, Letzel et al. 2000). The molecular weights of hetero-CHOs analyzed by MS are usually between 340 and 3000 Da, corresponding to DP 2 – DP ca. 17 (Figure 11.6 and Table 11.2).

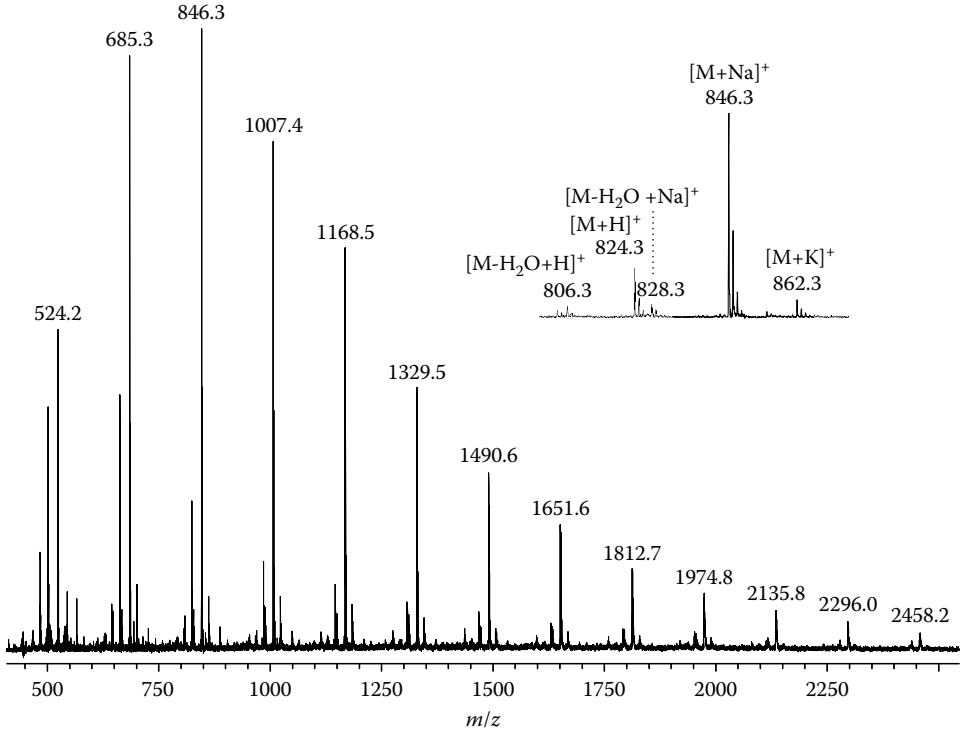


FIGURE 11.3 MALDI-TOF-MS (matrix: DHB) of a LMW fraction of D oligomers obtained after acid depolymerization of chitosan ($F_A < 0.01$) and fractionation by ultrafiltration and chromatography on Biogel P-2. Besides $[M + Na]^+$ ions of each oligomer of D_n ($n = 3 – 15$), protonated and potassiated species as well as elimination of H_2O are observed, as shown in the insert. (From Letzel, M.C. et al., *Adv. Chitin Sci*., 4, 545, 2000.)

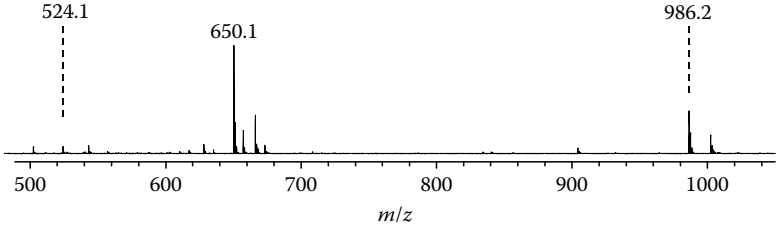


FIGURE 11.4 MALDI-TOF-MS (matrix THAP) of an equimolar mixture of D_3 (*m/z* 524 $[M + Na]^+$), A_3 (*m/z* 650 $[M + Na]^+$), and undeca-acetyl-D_3 (*m/z* 986 $[M + Na]^+$). Potassiated pseudomolecular ions are not labeled. Note that the intensity of *m/z* 524 is at the limit of detection (unpublished data from this laboratory).

11.4 APPLICATIONS OF MS FOR ANALYSIS OF MIXTURES OF HETERO-CHO OLIGOMERS AND HOMOLOGS

MS is used mostly for analyzing the composition of mixtures of CHOs produced by chemical or enzymatic depolymerization of chitosan, or by chemical, enzymatic, or chemoenzymatic synthesis (Table 11.3). Correlations of biological activity with average DP and F_A are described in several articles (for a review, see Kim and Rajapakse 2005). However, characterization of the mixtures by MS with respect to the presence of oligomers and homologs was done only in a few cases (Lee et al. 2003, Kittur et al. 2005, Torr et al. 2005, Cabrera et al. 2006, Ngo et al. 2008, Oliveira et al. 2008).

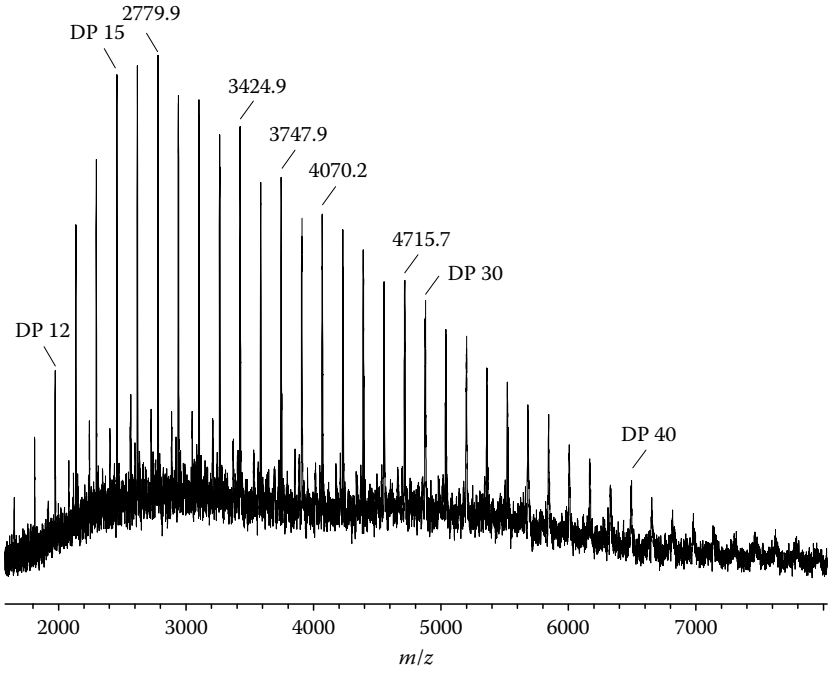


FIGURE 11.5 MALDI-TOF MS (matrix: THAP) of a MMW fraction of D oligomers obtained after acid depolymerization of chitosan ($F_A < 0.01$) and fractionation by ultrafiltration and chromatography on Biogel P-2. Labeled peaks refer to sodiated pseudomolecular ions. The highest oligomer detected at signal-to-noise ratio ≥ 2 is D_{43}. (From Letzel, M.C. et al., *Adv. Chitin Sci*., 4, 545, 2000.)

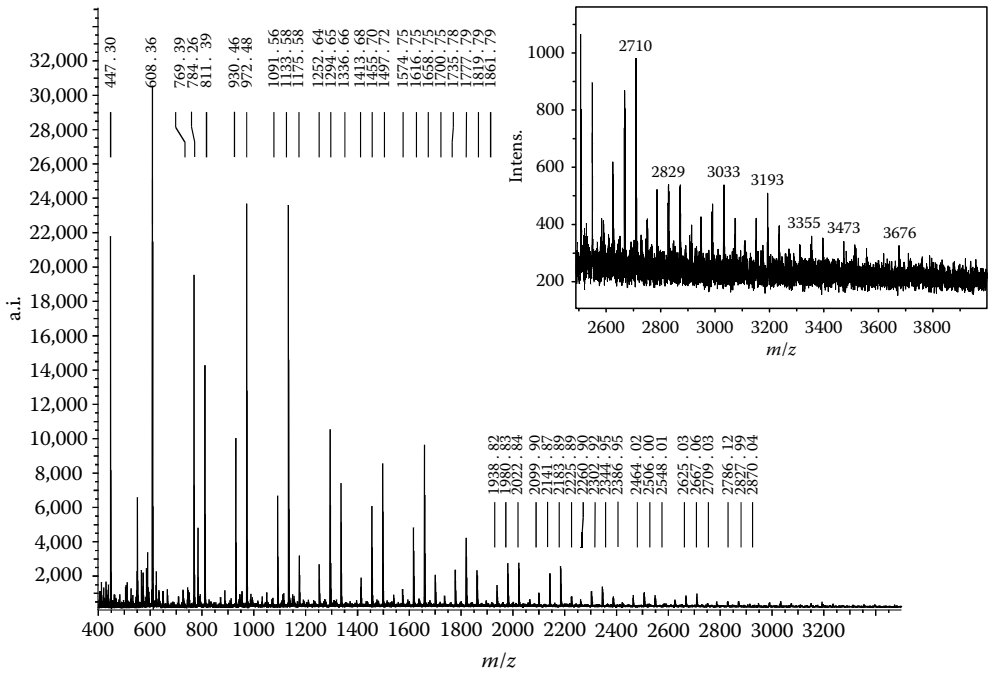


FIGURE 11.6 MALDI-TOF-MS (matrix DHB) of a mixture of hetero-CHO's obtained after enzymatic degradation of chitosan. The insert shows the enhanced spectrum in the mass range between 2500 and 3500 a.m.u. For peak assignment, see Table 11.2 (unpublished data from this laboratory).

TABLE 11.2
Assignment of Peaks and Relative Abundance (DA_2 = 100%) of Hetero-CHOs Detected by MALDI-TOF-MS in an Enzymatic Digest of Chitosan, Shown in Figure 11.6

DP	*y*	*z*	*m*/z Calcd	*m*/z Found	Relative Abundance (%)
2	0	2	447.16	447.31	71.25
3	1	2	608.23	608.35	100.00
4	2	2	769.30	769.39	63.95
4	1	3	811.31	811.39	46.73
5	3	2	930.37	930.46	32.85
5	2	3	972.38	972.48	77.52
6	4	2	1091.43	1091.56	21.86
6	3	3	1133.44	1133.58	77.29
6	2	4	1175.46	1175.58	10.49
7	5	2	1252.50	1252.64	8.79
7	4	3	1294.51	1294.65	34.55
7	3	4	1336.52	1336.66	24.28
8	6	2	1413.57	1413.69	6.23
8	5	3	1455.58	1455.70	19.92
8	4	4	1497.59	1497.71	28.01
8	3	5	1539.60	1539.72	3.04
9	7	2	1574.64	1574.75	4.11
9	6	3	1616.65	1616.74	15.73
9	5	4	1658.66	1658.75	31.57
9	4	5	1700.67	1700.75	6.79
10	7	3	1777.72	1777.78	7.78
10	6	4	1819.73	1819.79	13.85
10	5	5	1861.74	1861.79	7.68
11	8	3	1938.79	1938.83	4.83
11	7	4	1980.80	1980.84	8.99
11	6	5	2022.81	2022.84	9.09
11	5	6	2064.82	2065.85	2.18
12	7	5	2183.88	2183.89	8.41
12	9	3	2099.86	2099.89	3.37
12	6	6	2225.89	2226.87	2.71
13	10	3	2260.93	2260.91	2.17
13	9	4	2302.94	2303.93	3.68
13	8	5	2344.95	2344.94	4.55
13	7	6	2386.96	2387.93	2.68
14	10	4	2464.01	2464.99	2.97
14	9	5	2506.02	2507.00	3.49
14	8	6	2548.03	2548.99	2.93
15	11	4	2625.07	2625.04	2.03
15	10	5	2667.09	2668.03	2.84
15	9	6	2709.10	2710.03	3.21
15	8	7	2751.11	2751.88	1.37
16	12	4	2786.14	2787.08	1.71
16	11	5	2828.15	2829.08	1.77
16	10	6	2870.16	2872.06	1.76

(*continued*)

TABLE 11.2 (continued)
Assignment of Peaks and Relative Abundance (DA_2 = 100%) of Hetero-CHOs Detected by MALDI-TOF-MS in an Enzymatic Digest of Chitosan, Shown in Figure 11.6

DP	*y*	*z*	*m*/z Calcd	*m*/z Found	Relative Abundance (%)
16	9	7	2912.18	2914.01	1.30
17	13	4	2947.21	2948.09	1.40
17	12	5	2989.22	2991.08	1.54
17	11	6	3031.23	3033.11	1.76
17	10	7	3073.24	3074.38	1.24
18	13	5	3150.29	3151.08	1.38
18	12	6	3192.30	3193.11	1.67
18	11	7	3234.31	3236.11	1.29
19	14	5	3311.36	3312.00	1.08
19	13	6	3353.37	3355.29	1.17
19	12	7	3395.38	3396.02	1.16
20	15	5	3472.43	3473.25	1.12
21	15	6	3675.51	3675.88	1.07

Note: y = number of D residues, z = number of A residues, DP = $y + z$.

TABLE 11.3
Survey of the Literature on Analysis of CHOs and Some Derivatives by MS

CHO's D_yA_z Detected	Method of Preparation	MS Method	References
$y = 4 - 12$, $z = 0$	Transglycosylation with lysozyme, using D_3 and *N*,*N*′,*N*″-tri(monochloro) acetyl-D_3	MALDI (DHB)	Akiyama et al. (1995)
$y = 2 - 7$, $z = 0$	Hydrolysis of chitosan with a chitinase and *N*-acetylation of hetero-CHOs	FAB	Lopatin et al. (1995)
$y + z = 4 - 8$, $z = 0 - 3$	Synthesis of CHOs from oxazolines by transglycosylation with chitinase from *Bacillus* sp. or *Serratia marcescens*	MALDI (DHB)	Makino et al. (2006a)
$y + z = 3 - 17$, $z = 0 - 3$	Depolymerization of chitosan with a mixture of cellulase, α-amylase, and protease	MALDI (matrix not indicated)	Zhang et al. (1999)[a]
$y + z = 3 - 17$, $z = 0 - 3$	Depolymerization of chitosan with papain	MALDI (exp. conditions not given)	Lin et al. (2002)[b]
$y + z = 2–12$, $y = 0–3$	Depolymerization of chitosan with a pectinase isoenzyme from *Aspergillus niger*	MALDI (DHB)	Kittur et al. (2003)
$y + z = 3 - 16$, $z = 0 - 3$	Depolymerization of chitosan with HCl or with pectinase	MALDI (DHB)	Cabrera and Van Cutsem (2005)
$y + z = 3 - 13$, $y = 0 - 7$, $z = 1 - 6$	Depolymerization of chitosan with cellulase	MALDI (DHB)	Li et al. (2006a)
$y + z = 3 - 11$, $z = 0 - 6$	Depolymerization of chitosan with a protease	MALDI (DHB)	Li et al. (2005)[b]
$y + z = 2 - 9$, $y = 1 - 9$, $z = 0, 1$	Depolymerization of chitosan with protease from *Bacillus subtilis*	(−) ESI	Li et al. (2006b)

TABLE 11.3 (continued)
Survey of the Literature on Analysis of CHOs and Some Derivatives by MS

CHO's D_yA_z Detected	Method of Preparation	MS Method	References
$y + z = 2 - 6$, $z = 0 - 2$	Depolymerization of chitosan by sonolysis	ESI	Li et al. (2008)
$y + z = 2 - 12$, F_A 0.25 – 0.90, *m/z* 524 – 1623	CHO with controlled F_A by *N*-acetylation of D_y (y = 2–12)	MALDI (DHB)	Trombotto et al. (2008)
A_5, DA_4	Product analysis of chitin synthesis with recombinant NodC (*E. coli*)	FAB	Kamst et al. (1995); Samain et al. (1997)
D_y, $y = 1 - 3$	Analysis of D oligomers and catecholamine conjugates isolated from insect cuticle	ESI-MS and MSMS	Kerwin et al. (1999)[c]
$(DA)_n$, $n = \leq 7$	Synthesis of alternating DA oligomers from oxazoline donor by transglycosylation with chitinase from *S. marcescens*, *Streptomyces griseus*, and *Bacillus* sp.	MALDI (DHB)	Makino et al. (2006b)
D_3-M and A_5-M; M = 2,5-anhydro-D-mannose	Product analysis from depolymerization of chitosan with HNO_2	MALDI (DHB)	Tømmeraas et al. (2001)
[$^2H_3$-*N*-acetyl]-D_y, $y = 2 - 5$	Preparation from UDP-[$^2H_3$-*N*-acetyl]-D with chitin synthase from *Saccharomyces cerevisiae*	ESI	Becker et al. (2006)
[^{13}C-acetyl]-*O*-butyl D_2	N-[^{13}C]-acetylation of 1-*O*-butyl D_2	MALDI (DHB)	Yu and Prestegard, (2006)

[a] *Note:* Some peaks are incorrectly assigned, e.g., *m/z* 811 is $[M + Na]^+$ of DA_3 (not of D_2A_2, as stated in the chapter).
[b] Some peaks are mismatching.
[c] Also MS/MS spectra are reported. Note that fragment ions of D_3 are wrongly assigned.

Structure–bioactivity relations of hetero-CHO isomers are so far lacking, though appropriate tools are available and protein–ligand interactions have been studied on the molecular level (see Section 11.7).

Other applications to be mentioned are CI-MS for characterization of CHOs (DP < 30, $F_A = 0.2$) used to prepare liquid crystals (Dong et al. 2004), and MALDI-MS (DHB/oxalic acid) for an investigation of the mechanism of rapid deuterium exchange of amide protons in A4 (Price 2006).

Molecular mass determination by MS is also useful for analysis of substrate specificities and cleavage patterns of enzymes, including chitinases, chitosanases, lysozymes, and chitin deacetylases, as summarized with a few representative examples in Table 11.4. Continuous infusion of reaction mixtures into an ESI mass spectrometer (so called "real time" monitoring) was used to analyze the hydrolysis of D_z ($z = 4 - 6$) by several chitosanases and some mutants (Dennhart et al. 2008). ESI-MS is also suitable for simultaneous measurement of individual kinetic constants of enzymes in mixtures of substrates, as reported for a bacterial sulfotransferase with A_z ($z = 2 - 5$) (Pi and Leary 2004).

11.5 STRUCTURE ANALYSIS OF ISOMERS BY TANDEM MS

Because isomers (except anomers) do not exist for any homo-CHO, its structure is assigned directly from its molecular mass. In contrast, the molecular mass of a hetero-CHO reveals only its DP and F_A, leaving the determination of the sequence of D and A units as the final task, which must be handled by tandem MS. Fragment ion are generated by various dissociation methods (Budnik et al. 2003, 2006, Wolff et al. 2007), in most cases by CID. Ions leaving the ionization source are

TABLE 11.4
Survey of the Literature on Applications of MS for Investigations of Enzymes of Chitin Metabolism

Compounds Analyzed	Objectives	MS Method	Reference
D_yA_z, $y + z = 4 - 7$, $y = 0 - 7$, $z = 0 - 7$	Hetero-CHOs with controlled F_A by *N*-acetylation of D_y ($y = 4 - 7$) and inhibition of chitinases A and B from *S. marcescens*	MALDI (ATT, DHB, THAP)	Letzel et al. (2000)
D_yA_z, $y = 2 - 6$, $z = 0$	Cleavage pattern of endochitinase from *Vibrio carchariae*	LC ESI	Suginta et al. (2005)
D_yA_z, $y = 0, 1$, $z = 2 - 6$	Characterization of a chitin deacetylase from *V. cholerae*	ESI	Li et al. (2007)
D_yA_z, $y + z = 2 - 6$, $z = 0 - 6$	Characterization of chitin deacetylase from *Colletotrichum lindemuthianum*	ESI	Hekmat et al. (2003)
D_yA_z ($y = 1, 2$; $z = 3$)	Characterization and transglycosylation activity of a chitosanase from *Streptomyces griseus* HUT 6037	MALDI	Tanabe et al. (2003)
DAB and EDANS labeled D_1A_4	Cleavage patterns of chitinases	(+) and (−) LC ESI	Cottaz et al. (2000)
DAA, DDAA, DDA[a]	Cleavage pattern of a chitosanase from *Bacillus pumilus*	FAB	Fukamizo et al. (1994)
D_6, A_z ($z = 2, 3,4, 6$)	Characterization of extracellular chitinases from the parasitic fungus *Isaria japonica*	MALDI (THAP, NaI)	Kawachi et al. (2001)
D_3-MU, A_3-MU	Preparation of D_3-MU with chitin deacetylase from *C. lindemuthianum*, characterization of a chitosanase from *Streptomyces* sp. N174	FAB, MALDI (DHB)	Honda et al. (1999)
A_z, $z = 2 - 4$	Inhibition of a chitinase from *Aeromonas hydrophila* by polycyclic aromatic hydrocarbons	LC ESI	Ooki et al. (2007)
A_z, $z = 1 - 3$	Identification of hydrolysis products of glycol chitin with chitinase from *Papaya carica*	LC ESI	Chen et al. (2007)
A_2-*p*NP, DA-*p*NP	Preparation of DA-*p*NP from A_2-*p*NP with chitin deacetlyase from *C. lindemuthianum* and characterization of chitinases	FAB	Tokuyasu et al. (1999)
A_2	Characterization of an exochitinase from baculovirus *Epiphyas postvittana nucleopolyhedrovirus*	ESI	Young et al. (2005)
N,*N*′-diacetylchitobionoxime-*N*-phenylcarbamate (HM508)	Inhibition of chitinase B from *S. marcescens*	ESI	Vaaje-Kolstad et al. (2004)
Fuc-A_4, Gal-A_3	Study of substrate specificity of family 18 chitinases from *Trichoderma harzianum*	MALDI (DHB)	Boer et al. (2004)

[a] The sequences are confirmed by ^{13}C NMR spectroscopy.

accelerated by a voltage electric potential, typically from 15 to 50 V, and guided into a collision cell where they collide with an inert gas, usually argon. The decrease in kinetic energy results in cleavage of bonds with retention of the charge in any of the fragments. These product ions are recorded in a second mass spectrum. In MS^3, a particular product ion is selected and fragmented again. Principally, sequential product ions generated by MS^3 can be further fragmented to give MS^4 data. In MALDI-PSD MS, product ions are formed by spontaneous decay of ions after ionization and acceleration (Spengler 1997).

Fragment ions generated via MS^n experiments of CHOs are named according to the nomenclature developed for oligosaccharides and glycoconjugates (Figure 11.7) (Domon and Costello 1988). For example, cleavage of a glycosidic bond between an anomeric carbon and the glycosidic oxygen and retention of the charge on the aglycon results in a B-type ion.

The numbering of sugar units is from left to right for (A)-, (B)-, and (C)-type ions, and from right to left for (X)-, (Y)-, and (Z)-ions. X-ions formed by fragmentation of the reducing-end sugar residue are numbered as ${}^{p,q}X_{n-1}$, where p and q are the numbers of ring atoms next to which the bond is broken, and n is the number of the sugar, counting from the reducing end. ${}^{1,5}X_{n-1}$ and ${}^{O,2}X_{n-1}$ ions, respectively, were observed in FAB MS (Bosso and Domard 1992) and in tandem FAB MS of homo-CHOs (Singh et al. 1995). Otherwise, ${}^{1,5}A_n$-, X_{n-1}, and Z_n-ions are rarely observed and are not further considered here. The most commonly observed fragmentation processes are summarized in Table 11.5.

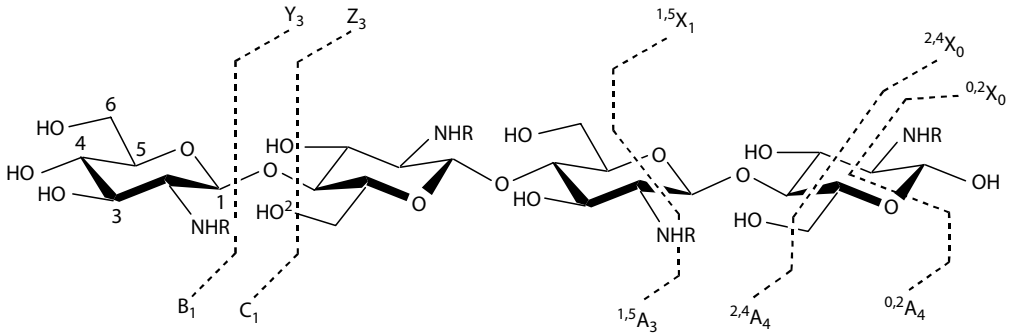


FIGURE 11.7 Nomenclature of fragment ions of a tetra-CHO. (From Domon, B. and Costello, C.E., *Glycoconj. J.*, 5, 397, 1988.)

TABLE 11.5
Fragmentation Processes Ions Commonly Observed in MS of Oligosaccharides

Type of Ion	Cleaved Bond	Charged Fragment
B_n	Glycosidic C(1)–O(1)	Glycon
Y_n	Glycosidic C(1)–O(1)	Aglycon
C_n	Glycosidic O(1)–C(4)	Glycon
Z_n	Glycosidic O(1)–C(4)	Aglycon
${}^{O,2}A_n$	Cross-ring O(5)–C(1) and C(2)–C(3)	Glycon
${}^{O,2}X_{n-1}$	Cross-ring O(5)–C(1) and C(2)–C(3)	Aglycon
${}^{2,4}A_n$	Cross-ring C(2)–C(3) and C(4)–C(5)	Glycon
${}^{2,4}X_{n-1}$	Cross-ring C(2)–C(3) and C(4)–C(5)	Aglycon
${}^{1,5}A_n$	Cross-ring C(1)–C(2) and C(5)–O(5)	Glycon
${}^{1,5}X_{n-1}$	Cross-ring C(1)–C(2) and C(5)–O(5)	Aglycon

B-type ions are most abundant in ESI-MS/MS spectra of protonated molecules of CHOs whereas sodiated molecules dissociate also by ^{O,2}A- and ^{2,4}A-type cleavage (cf. Budnik et al. 2003). A strong complexation with an alkali metal ion, e.g., Li^+, gives a higher degree of cross-ring fragmentation than a weakly bound ion, e.g., Cs^+ (Cancilla et al. 1999). Cross-ring fragmentation was also observed by ESI-MS in a study on the association of D_4 and A_4 with copper(II) ions, revealing strong complexation with D_4 but not with A_4 (Shabgholi et al. 1997). Other, usually nondiagnostic fragment ions can be generated by loss of ammonia (–17 Da), water (–18 Da), ketene (–42 Da), and/or acetamide (–59 Da). Fragmentation of molecular ions was also observed in MS^1 spectra generated by FAB (Bosso et al. 1986, Bosso and Domard 1992), ESI (Shabgholi et al. 1997), and MALDI (Trombotto et al. 2008).

To illustrate the fragmentation of a hetero-CHO, we consider the most commonly observed A-, B-, C-, and Y-type ions of the six isomers of D_2A_2 (Issaree 2008, Vijayakrishnan 2008) (Table 11.6). Several fragments appear with the same mass, i.e., they are isobaric, but result from different fragmentation processes and sequences. For example, the $^{2,4}A_4$ fragment of a reducing-end A residue of D_2A-A (calcd. *m/z* 608 [M + Na – 161]), could also represent C- or Y-ions formed by loss of a neutral D-unit, either from the reducing end of DA_2-D or the nonreducing end of D-DA_2, respectively. Obviously, sequencing of CHO's by MS/MS may give ambiguous results, especially when mixtures containing isomers in varying concentrations must be analyzed and MS^n is not available. As an example, FT-ICR ESI CID MS/MS and MS^3 spectra of ADAD are shown in Figures 11.8 (protonated) and 11.9 (sodiated), respectively. The occurrence of intensive B-type ions in MS/MS of *m/z* 747 $[M + H]^+$ as well as in MS^3 of *m/z* 568 confirms the sequence of ADAD (Figure 11.8). Note that *m/z* 365 could principally represent isobaric B_2- and Z_2-ions. However, the high intensity of *m/z* 365 confirms the B_2-ion. MS^3 of *m/z* 365 gives the very intensive B_1 ion of a nonreducing end A residue at *m/z* 204, which would not be possible when *m/z* 365 would be the Z_2 ion. Except the diagnostic Y_3-ion at *m/z* 544, other Y-type ions are barely detectable. C-type ions appear with very low intensity at *m/z* 222 and 383, the latter being isobaric with Y_2. CID MS/MS of the sodiated molecular ion, *m/z* 769, shows a low intensity B_2-ion at *m/z* 387 and stronger B_3- and C_3-ions at *m/z* 590 and 608, respectively (Figure 11.9). The most intensive cross-ring fragment is the DA_2-$^{O,2}A_4$-ion at *m/z* 710, which is diagnostic for a reducing-end D residue. If an A residue would be at the reducing end, *m/z* 608 could be the corresponding D_2A-$^{O,2}A_4$-ion, which is excluded because a corresponding D_2A-B_3-ion (calcd. *m/z* 548) is not observed in the MS^3 spectrum.

TABLE 11.6
Calculated Mono-Isotopic Masses of Sodiated Fragment Ions of D_2A_2 Isomers

***m/z* 769 $[M + Na]^+$**	**AADD**	**ADAD**	**ADDA**	**DAAD**	**DADA**	**DDAA**
$^{O,2}A_4$	710.26	710.26	668.25	710.26	668.25	668.25
$^{2,4}A_4$	650.24	650.24	608.23	650.24	608.23	608.23
B_3	590.22	590.22	548.21	590.22	548.21	548.21
C_3	608.23	608.23	566.22	608.23	566.22	566.22
Y_3	566.22	566.22	566.22	608.23	608.23	608.23
***m/z* 747 $[M + H]^+$**	**AADD**	**ADAD**	**ADDA**	**DAAD**	**DADA**	**DDAA**
B_3	568.24	568.24	526.23	568.24	526.23	526.23
C_3	586.25	586.25	544.24	586.25	544.24	544.24
Y_3	544.24	544.24	544.24	586.25	586.25	586.25
B_2	407.17	365.16	365.16	365.16	365.16	323.15
C_2	425.18	383.17	383.17	383.17	383.17	341.16
Y_2	341.16	383.17	383.17	383.17	383.17	425.18
B_1	204.09	204.09	204.09	162.08	162.08	162.08
C_1	222.10	222.10	222.10	180.09	180.09	180.09
Y_1	180.09	180.09	222.10	180.09	222.10	222.10

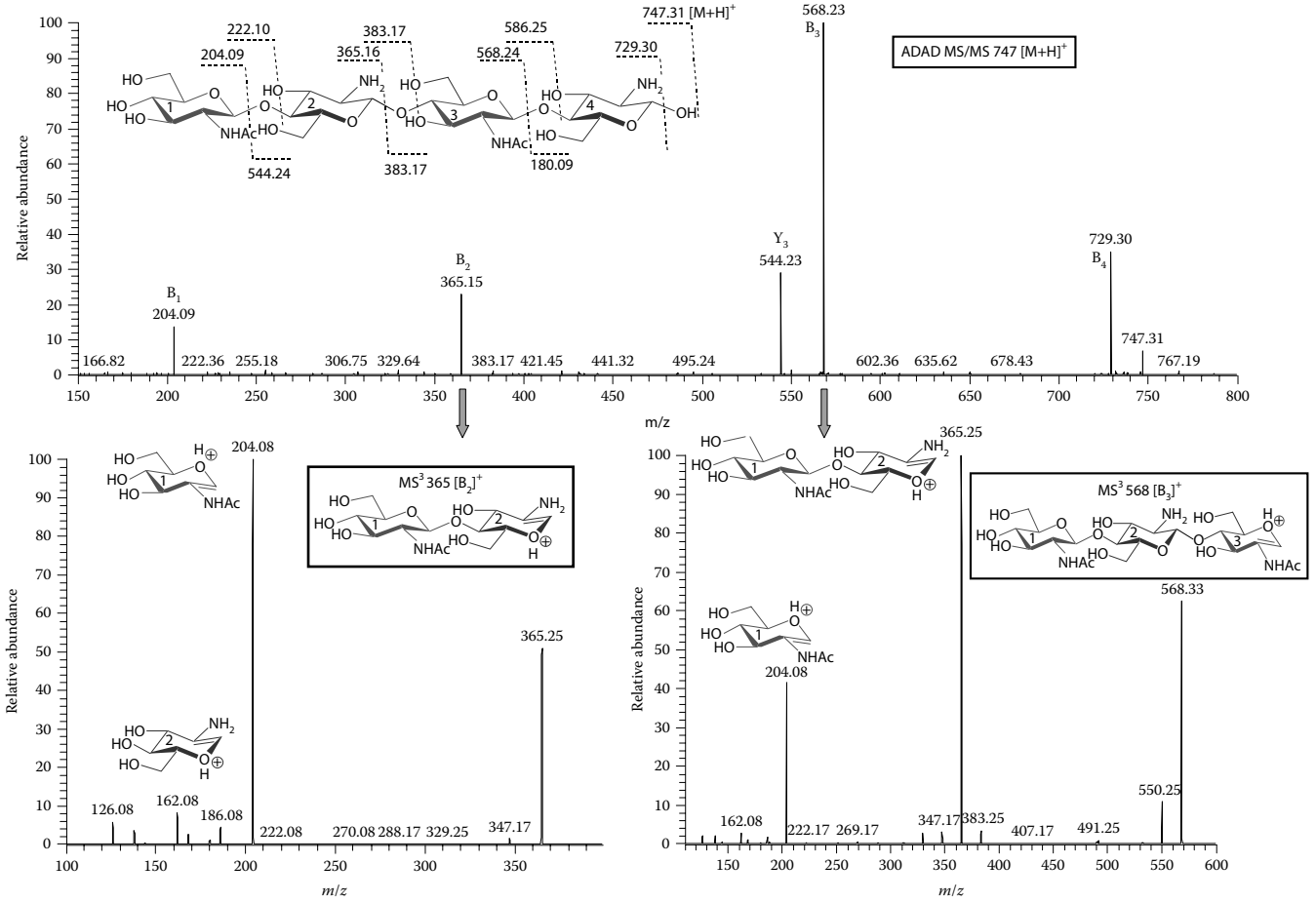


FIGURE 11.8 Positive ion (H^+) FT-ICR ESI-MS^n spectra of ADAD. The MS/MS spectrum shows a series of intensive B_n-type ions at *m/z* 204, 365, and 568, besides the Y_3-ion at *m/z* 544. The B_2- and B_3-ions are fragmented by MS^3 to give daughter B-ions as the most intensive peaks. (From Issaree, A. et al., Mass spectrometry of aminoglucan oligosaccharides using electrospray ionization MS/MS and MS/MS/MS, *Proceedings of the 9th International Conference of the European Chitin Society* (*EUCHIS '09*), Venice, Italy, 2009.)

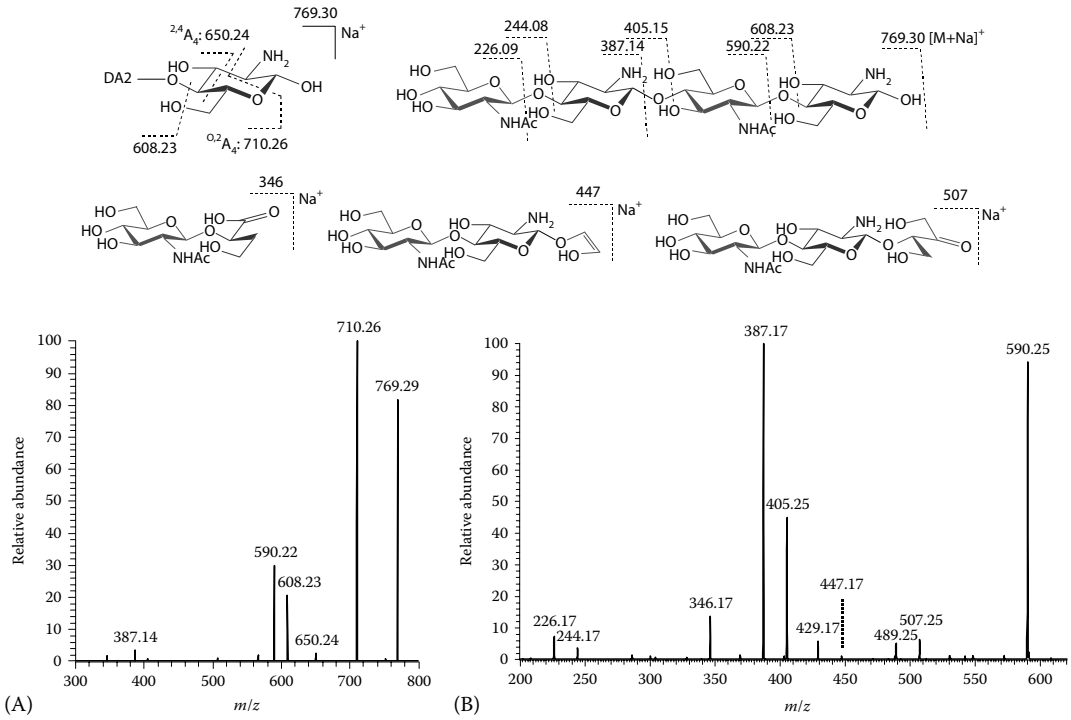


FIGURE 11.9 Positive ion (Na^+) FT-ICR ESI CID MS^n spectra of ADAD. (A) MS/MS shows cross-ring $^{O,2}A_4$-fragmentation of the reducing end D-residue by a strong peak at *m/z* 710 and a very weak peak at *m/z* 650. C_3-, B_3- and B_2-ions are detected at *m/z* 608, 590 and 387, respectively. (B) MS^3 of *m/z* 608 gives fragments at *m/z* 387 (B_2 of B_3) and 226 (B_1 of B_3), as well as $^{O,2}A_n$-cross-ring fragments at *m/z* 346 and 507 and the $^{2,4}A_3$-ion at *m/z* 447. The peak at *m/z* 429 accounts for loss of water $[^{2,4}A_3+Na-18]^+$. (From Issaree, A. et al., Mass spectrometry of aminoglucan oligosaccharides using electrospray ionization MS/MS and MS/MS/MS, *Proceedings of the 9th International Conference of the European Chitin Society* (*EUCHIS '09*), Venice, Italy, 2009.)

11.6 REDUCING-END TAGGING FOR SEQUENCE ANALYSIS

Sequence analysis by MS/MS is straightforward when at least one of the sugar residues is linked to a unit, which is different from D or A, as is the case in Nod factors (for a review, see Van der Drift et al. 1998) and related CHOs (Olsthoorn et al. 1998, Haslam et al. 1999, Natunen et al. 2001, Pacios-Bras et al. 2002). Hetero-CHOs can be sequenced by multistep enzymatic procedures, employing hexosaminidases and *N*-acetylhexosaminidases, which specifically cleave D or A residues from the nonreducing end of the oligosaccharide chain, followed by MS analysis of the resulting oligosaccharide, which is shorter by one or more sugar units (Izume et al. 1992, Mitsutomi et al. 1996, 1997, 1998).

The sequence of an underivatized hetero-CHO can be deduced from the occurrence of $^{O,2}A_n$ and $^{2,4}A_n$ cross-ring fragments in MS^2 of the pseudomolecular ion as well as MS^3 of sodiated C-type ions (unpublished results from this laboratory). In this case, the sodium ion may be understood as a "tag," which allows identification of the reducing end sugar.

Derivatives of reducing sugars are obtained by reaction with a suitable reagent, which introduces a "tag" at the reducing end. All Y-type ions generated in tandem MS retain the tag and are identified by the mass increment of the reagent. This technique is frequently used in glycobiology but has rarely been applied to the analysis of CHOs. As compared with MS^n of underivatized oligosaccharides, end-group tagging requires an additional step in the analytical protocol, including clean-up of the sample by chromatography and/or charcoal adsorption (Haebel et al. 2007).

Reduction of aldoses or ketoses with sodium borohydride in water is widely used in glyco-analysis, including *N*-glycans of glycoproteins. The reaction can be done easily with microgram amounts of an oligosaccharide. Desalting of the sample is required before analysis (for a typical protocol, see e.g., Kawasaki et al. 2003). The resulting oligosaccharide-alditols as well as Y-type fragment ions are detected by a mass increment of 2 Da. However, there is no improvement in sensitivity because, as in oligosaccharide-aldoses, Y-type ions of oligosaccharide-alditols will appear with lower intensity than B-type ions. Reduction of CHOs to alditols was also achieved on a preparative scale by catalytic hydrogenation with a ruthenium catalyst, followed by FAB-MS of the products (Nagae et al. 1992).

Acid-catalyzed reaction of CHOs with methanol gives methyl glycosides. This reaction has been used to identify and sequence DA, DDA, and DDDA after of hydrolysis of chitosan with a chitosanase from *Aspergillus fumigatus* by ESI-MS/MS (Cheng et al. 2006) and DDA and ADDA, which had been separated from a noncovalent protein–ligand complex of CHOs with a chitosanase from a fungus, *Paecilomyces lilacinus* (Chen et al. 2005). Like in alditols and free oligosaccharides, B-type ions dominate in the MS/MS of methyl glycosides and Y-type ions appear with lower intensity but are identified unequivocally by a mass increment of 14.

Formation of a hydrazone with Girard's Reagent T introduces a permanent positive charge into the molecule. Na^+ and K^+ adducts are suppressed, thus increasing the sensitivity of detection (Naven and Harvey 1996). This reaction has been applied to CHOs but does not offer a major improvement in comparison with reductive amination (unpublished results from this laboratory).

The reaction of a reducing sugar with an amine in the presence of sodium cyanoborohydride is named reductive amination (Figure 11.10). Tagging with ABN (Schwaiger et al. 1994) has not yet been applied to CHOs. Most suitable are heteroaromatic polycyclic amines, such as amac (Okafo et al. 1997, Bahrke et al. 2002) and aa-Ac (Charlwood et al. 2000, Haebel et al. 2007) (Table 11.7). In positive mode MS/MS, the charge is retained mostly on the heteroaromatic ring system. The spectra appear generally with a series of characteristic Y-type ions from which the sequence is easily deduced, as shown for a mixture of two isomeric D_3A_3-amac derivatives in Figure 11.11 (Bahrke et al. 2002). Exceptions are possible, as evident from the rather intensive peak for the ion of *m/z* 912 $[B_5\text{-}(D_3A_2) + Na]^+$. Tagging with amac has also been used for sequence analysis of hetero-CHOs that were resistant to hydrolysis by chitinase B from *Serratia marcescens* and thus inhibit the enzyme, as well as of transglycosylation products (Cederkvist et al. 2008).

The conversion of an oligosaccharide to the glycosylamine, followed by transamidation with [2,5-dioxo-1-pyrrolidinyl 4-([2,2′:6′,2″-terpyridin]-4′-yl-κN1,κN1′,κN1″)benzoate](2,2′:6′,2″-terpyridine-κN1,κN1′,κN1″)-ruthenium-(II) chloride introduces a heavy metal tag at the reducing end. Ions retaining the tag are doubly charged and identified by the characteristic isotope pattern of ruthenium, as shown by detection of Ru-(II) tagged *N*,*N*″-diacetylchitobiose in an enzymatic digest of glycosylamines of A_z ($z = 3 - 5$) with a thermophilic bacterial chitinase (Ito et al. 2009).

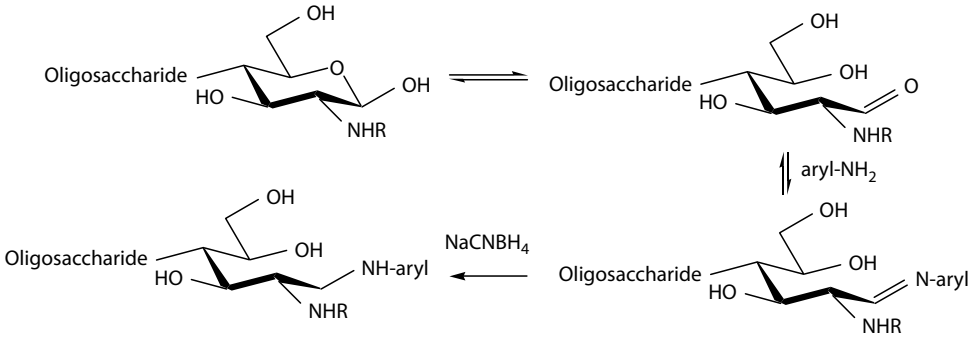
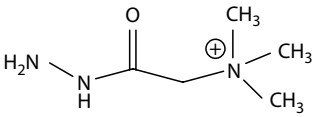
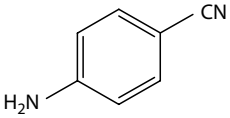
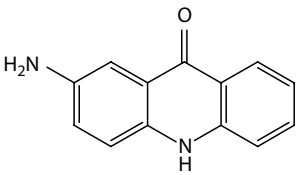
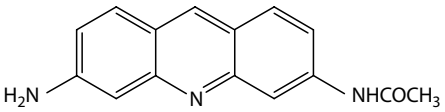


FIGURE 11.10 Reductive amination. The reducing end hemiacetal is in equilibrium with a small amount of the aldehyde which reacts with the amine to form a Schiff base. The final step, i.e., in situ reduction of the Schiff base to the 1-deoxy-1-arylaminoalditol, is irreversible and drives the equilibrium.

TABLE 11.7
Reagents for Reducing-End Tagging of Oligosaccharides

Tag	Formula of Reagent	Name of Reagent	Mass Increment
Reduction			
Alditol	$NaBH_4$	Sodium borohydride	2.0156
Glycosidation			
Me	CH_3OH	Methanol	14.0157
Formation of hydrazones			
		Carboxymethyltrimethylammonium chloride hydrazide (Girard's T reagent)	114.1032
Reductive amination (*amine + NaCNBH*$_4$)			
ABN		4-Aminobenzonitrile	102.0582
amac		2-Aminoacridone	194.0844
aa-Ac		3-(Acetylamino)-6-aminoacridine	235.1110

11.7 ANALYSIS OF NONCOVALENT PROTEIN–LIGAND COMPLEXES

The analysis of complexes of a fungal elicitor of plant chitinases by ESI-MS with A_z ($z = 1 – 6$) revealed increasing binding affinities for oligomers of DP > 2 (van den Burg et al. 2004). A noncovalent enzyme–CHO complex was observed for the first time by online ESI-MS analysis of the hydrolysis of A_6 with lysozyme, showing the association of the substrate A_6 as well as the products A_3 and A_4 with the enzyme (Ganem 1991). The catalytic proton transfer, which initiates acid-catalyzed cleavage of glycosidic bonds, occurs even in the gas phase, as was observed by ESI-MS of substrate complexes of lysozyme with A_z ($z = 4 – 8$), but not of nonsubstrate complexes with malto-oligomers (He et al. 1999). "Real-time" monitoring yielded data on the kinetics of the reaction (Dennhart and Letzel 2006).

Competitive inhibition by nonproductive binding of a hetero-chitopentasaccharide to a chitinase was discovered by top-down nano-ESI-MS/MS via CID of the noncovalent complex of chitinase B from *S. marcescens* with DADAA (Cederkvist et al. 2006). The enzyme belongs to family 18 glycosidases, hydrolyzing chitin, chitosan, and CHOs by substrate-assisted catalysis to give specifically a reducing-end A residue. Further, the enzyme removes disaccharides from the nonreducing end of the oligo- and polysaccharide chain and shows a high preference for binding of an A residue in the first neighboring position (Horn et al. 2006). The next neighbor in DADAA is a D residue, which binds at the catalytic site but cannot be cleaved because the lack of substrate-assisted catalysis. Thus, nonproductive binding of DADAA to the chitinase results in enzyme inhibition.

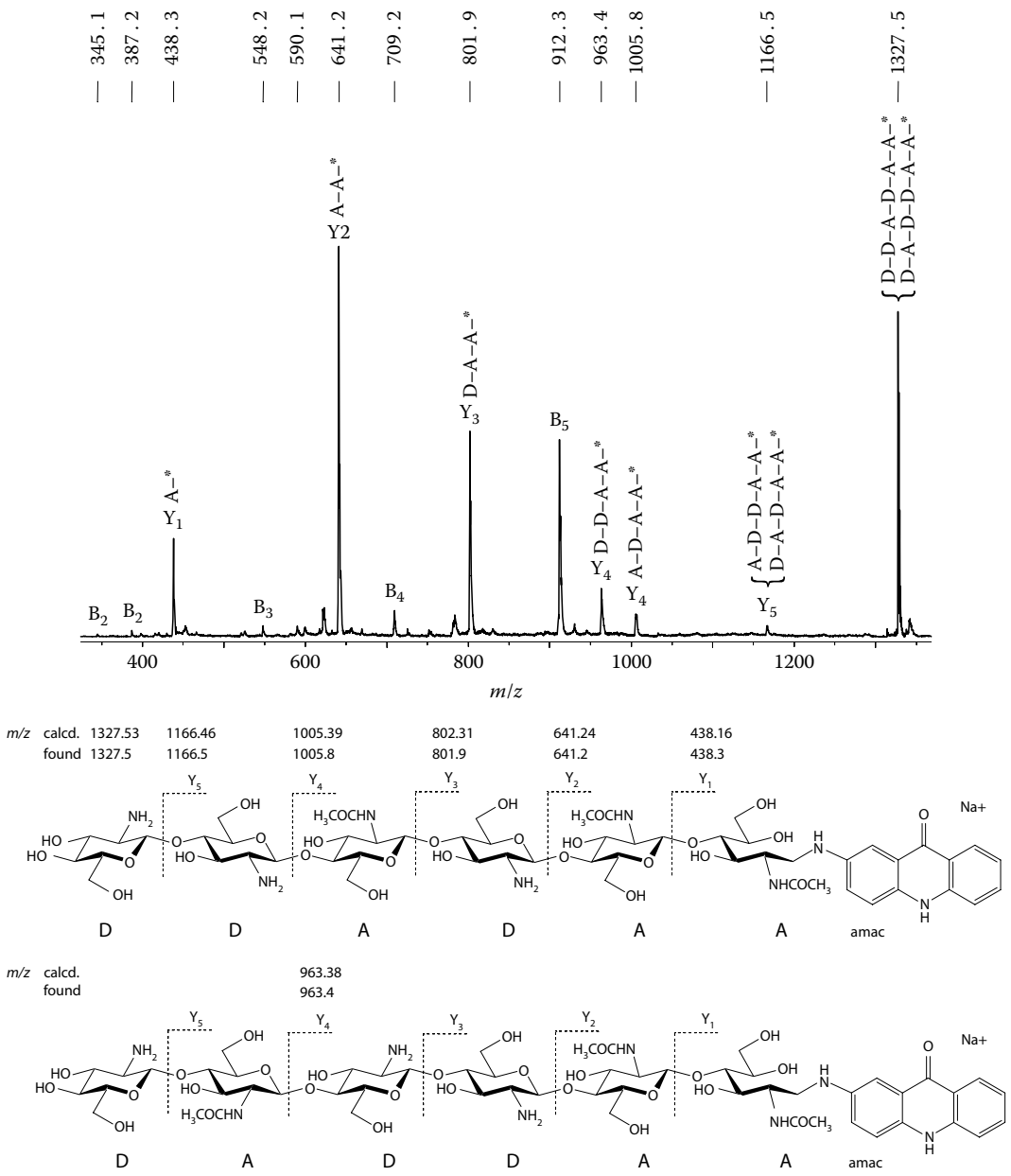


FIGURE 11.11 MALDI TOF PSD MS of amac tagged D_3A_3 (*m/z* 1327 $[M + Na]^+$) obtained by reductive amination of CHO's isolated from an enzymatic digest of chitosan. (From Bahrke, S. et al., *Biomacromolecules*, 3, 696, 2002.)

11.8 OUTLOOK

In order to understand the remarkable biological activities of CHOs (Kim and Rajapakse 2005) and create a basis for developing novel therapeutics for e.g., treatment of parasitic infections, asthma, and certain forms of rheumatism (Einarsson et al. 2003, Cederkvist et al. 2008), it is mandatory to characterize mixtures of CHOs with respect to their composition of oligomers, homologs, and isomers. MS is the ideal, if not the only tool to handle this task. The unavailability of sophisticated and expensive instrumentation in many laboratories can be overcome by using analytical services,

which are offered around the world. More important for establishing structure–bioactivity relations is an understanding of carbohydrate chemistry, in particular isomerism in hetero-CHOs. We hope that this review will contribute to future progress in this area.

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12 The Use of Various Types of NMR and IR Spectroscopy for Structural Characterization of Chitin and Chitosan

Mohammad Reza Kasaai

CONTENTS

12.1 INTRODUCTION

Oligomers and polymers of chitin and chitosan are homo-polymers or hetero-polymers of two monosaccharides [β-(1-4)-2-acetamido-2-deoxy-D-glucose (GlcNAc); and β-(1-4)-2-amino-2-deoxy-D-glucose (GlcN)], which are joined by glycosidic linkages. Oligosaccharides are generally defined as carbohydrate polymers containing a few monomer residues. The borderline between oligosaccharides and polysaccharides is not precise. The number of monomer units for oligosaccharides have

been reported to be up to 10 or 20 monomer residues. The word "chitosan" is used for both partially and completely deacetylated chitosans. The chemical structure of chitin is similar to that of cellulose. The difference between the chemical structure of cellulose and that of chitin is that the 2-hydroxy groups of cellulose have been replaced with *N*-acetyl groups. The chemical structures of cellulose, fully *N*-acetylated chitin, and completely *N*-deacetylated chitosan are illustrated in Figure 12.1. However, fully *N*-acetylated chitin and completely *N*-deacetylated chitosan are not commercially available. The degree of *N*-acetylation (DA) has been employed to differentiate chitin from chitosan.

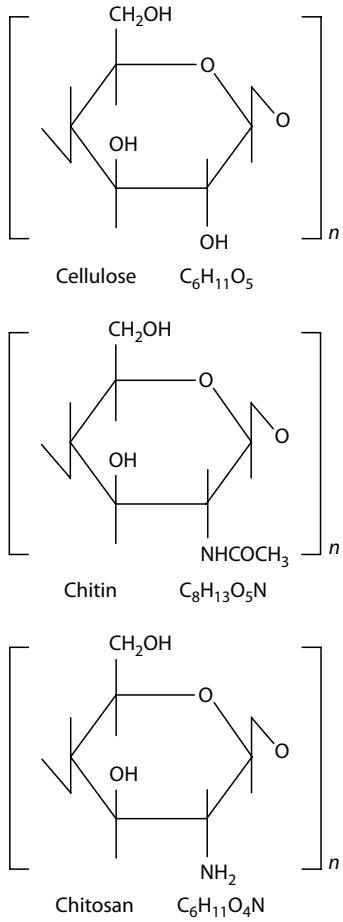

FIGURE 12.1 Chemical structures of cellulose, chitin, and chitosan.

Oligomers and polymers of chitin, chitosan, and their derivatives are extensively used in a broad range of fields that include agriculture, food, textile, cosmetics and medicine (Sandford and Hutchings 1987, Sandford 1989, Sandford and Steinnes 1991, Shahidi et al. 1999, Chatelet et al. 2001). They find vast applications because of their numerous interesting biological, physicochemical, and mechanical properties (Domard et al. 1998, Uragami et al. 2001). Their properties are closely related to their chemical structures, composition, sequence, DA, and molecular size. An insight into the biological and physiological functions of chitin and chitosan at the molecular level require a precise knowledge on the primary and secondary structures. Elucidating their structure helps our understanding of their biological roles. The complete structural characterization of the oligomers and polymers involves determining (1) the type, number, and primary sequence of the constituting monomers residues, including the occurrence of branch point in block copolymers and the location of amine and *N*-acetyl groups and (2) their three-dimensional (3D) structure. Although determining the complete structure of a carbohydrate usually requires the application of a combination of chemical, physical, enzymatic, and spectroscopic methods.

IR and NMR spectroscopy are relatively easy-to-apply for a qualitative and comparative evaluation of the chemical structure and the DA determination. These techniques are nondestructive methods and do not need initial treatment such as hydrolysis, pyrolysis, and derivatization. This chapter describes the structural characterization of chitin and chitosan (as oligomers and polymers) by IR, near-IR, and various types of NMR spectroscopy techniques. This study provides information on (1) composition, sequence, and type of residues; and (2) any structural changes occurring in the molecules as a result of different processes (degradation, deacetylation, and acetylation). The influences of acids, alkali, moisture, and impurities on the NMR and IR spectra of the original molecules will be also discussed.

12.2 DESCRIPTION OF DIFFERENT TECHNIQUES FOR STRUCTURAL CHARACTERIZATION AND THE DA DETERMINATION

The chemical structure and the DA determined by various IR and NMR techniques are described as follows: (1) the DA of chitin/chitosan by various types of NMR (^{1}H NMR, ^{13}C NMR, and ^{15}N NMR) and IR (IR and NIR) have been determined as described in the following: determination of the ratio of A_p/A_R: where A_p is height or area of the probe signal; and A_R is the height or area of reference

signal. The DA of unknown samples is estimated by comparing the values of A_p/A_R with a calibration curve. The calibration curve is constructed by plotting the ratio of A_p/A_R versus DA, where the DA determined by the same spectroscopy technique or by a reference and validate method such as ^{1}H NMR spectroscopy. The specific description for each technique has been appeared in the individual techniques as follows.

12.2.1 ^{1}H NMR Spectroscopy

Dilute solutions of chitosan samples in an aqueous acid (CD_3COOD/D_2O or DCl/D_2O) were prepared and their ^{1}H NMR spectra were recorded between 0 and 10 ppm using a proton NMR spectrometer (Hirai et al. 1991, Värum et al. 1991a, Rinaudo et al. 1992). Different models of proton NMR spectrometer (fabricated by Bruker Company) have been employed to record the spectra.

In most of reports, the protons of CH_3 correspond to *N*-acetyl group have been selected as probe nucleus/nuclei. The height or area of the signal is proportional to the number of protons. The DA has been determined using Equations 12.1 through 12.4 (Hirai et al. 1991, Rinaudo et al. 1992, Tan et al. 1998, Kasaai et al. 1999):

$$\mathrm{DA} = \frac{(1/3 \times I_{\mathrm{CH_3}})}{\left(1/6 \times I_{(\mathrm{H_2}-\mathrm{H_6})}\right)} \times 100 \tag{12.1}$$

where

I_{CH_3} is the intensity of CH_3

$I_{(H_2-H_6)}$ is the summation intensities of H_2%, H_3%, H_4%, H_5%, and H_6%

$$\mathrm{DA} = \frac{7\left[I_{\mathrm{H_1(GcNAc)}} + I_{\mathrm{CH_3(GlcNAc)}}\right]}{4\left[I_{\mathrm{H_1(GlcN)}} + I_{\mathrm{H_2(G1cNAc)}}\right] + I_{\mathrm{CH_3(G1cNAc)}}} \times 100 \tag{12.2}$$

$$\mathrm{DA} = \frac{I_{\mathrm{CH_3}}}{\left(3 \times I_{\mathrm{H\text{-}1\,proton}}\right)} \times 100 \tag{12.3}$$

$$\mathrm{DA} = \frac{I_{\mathrm{CH_3}}}{\left(3 \times I_{\mathrm{H\text{-}2\,proton}}\right)} \times 100 \tag{12.4}$$

12.2.2 ^{13}C NMR Spectroscopy

^{13}C NMR spectra of chitin/chitosan in solid state have been recorded (Raymond et al. 1993). A cross-polarization magic-angle-spinning carbon NMR (CP/MAS ^{13}C NMR) has been used to record the spectra of chitin/chitosan samples (Värum et al. 1991b, Raymond et al. 1993, Ottøy et al. 1996, Heux et al. 2000, Duarte et al. 2001a,b). The CP/MAS ^{13}C NMR spectrometer equipped with high-power ^{1}H decoupling (HPDEC) has been also employed to record ^{13}C NMR spectra (Heux et al. 2000, Duarte et al. 2001a). The procedure of Ottøy et al. (1996) has been modified with changes in relaxation delay time and contact time (Tolaimate et al. 2000, Duarte et al. 2001b). The contact and delay time are the most variable parameters to record the CP/MAS ^{13}C NMR spectrum (Tolaimate et al. 2000, Duarte et al. 2002, Guinesi and Cavalheiro 2006). The DA of the samples was calculated from the intensity of *N*-acetyl carbon atom divided by the summation intensities of carbon atoms of the D-glucopyranosyl ring as follows (Raymond et al. 1993, Ottøy et al. 1996, Tolaimate et al. 2000, Duarte et al. 2002):

$$\mathrm{DA} = \frac{I_{\mathrm{N-CH_3}}}{\left[1/6\left(I_{\mathrm{C_1}} + I_{\mathrm{C_2}} + I_{\mathrm{C_3}} + I_{\mathrm{C_4}} + I_{\mathrm{C_5}} + I_{\mathrm{C_6}}\right)\right]} \tag{12.5}$$

12.2.3 ^{15}N NMR Spectroscopy

^{15}N NMR spectra of chitin/chitosan in solid state have been recorded between 0 and 200 ppm using NMR spectrometer operating at 30 or 200 MHz (Yu et al. 1999, Heux et al. 2000). Cross polarization and strong magnetic fields were used to increase resolution and sensitivity. The different contact times were employed to minimize errors for quantitative evaluation of the DA (Yu et al. 1999, Heux et al. 2000). Two peaks have been recognized in the spectra of chitin/chitosan, and the DA was calculated according to

$$\mathrm{DA} = \frac{\left(I_{N\text{-acetyl group}}\right)}{\left(I_{N\text{-acetyl group}} + I_{\text{amine group}}\right)} \tag{12.6}$$

where the two peaks correspond to amine and *N*-acetyl groups.

12.2.4 Infrared Spectroscopy

The IR region of an electromagnetic spectrum extends from 0.8 to 400 μm. The range of 0.8–2.5 μm (12,500–4,000 cm^{-1}) is generally considered to be near-IR region; from 2.5 to 25 μm (4000–400 cm^{-1}) is known as mid-range region; and from 25 to 400 μm (400–25 cm^{-1}) is as far-IR region. IR spectra generally recorded in the range of 1200–4000 cm^{-1}. The sample was prepared as a thin film made from a mixture of KBr and chitin/chitosan or as a thin film made from casting procedure of chitin/chitosan solution. Several absorption band ratios such as A_{1560}/A_{2878}, A_{1655}/A_{2878}, A_{1655}/A_{3450}, A_{1320}/A_{3450}, A_{1560}/A_{2878}, A_{1655}/A_{1070}, A_{1655}/A_{1030}, A_{1560}/A_{1160}, A_{1560}/A_{897}, and A_{1320}/A_{1420} have been proposed for determination of the DA (Sannan et al. 1978, Miya et al. 1980, Muzzarelli et al. 1980, Domszy and Roberts 1985, Baxter et al. 1992, Shigemasa et al. 1996a,b, Brugnerotto et al. 2001a, Dong et al. 2002). In addition, the DA of chitin/chitosan samples has been also determined by evaluation of several absorption band ratios using a statistical method (Duarte et al. 2002). A detailed description on the various procedures of IR technique has been recently published (Kasaai 2008).

12.2.5 Near Infrared (NIR) Spectroscopy

Near-IR (NIR) has been recorded from 1100 to 2500 nm (9090–4000 cm^{-1}) and their second derivatives spectra have been used to determine the DA (Rathke and Hudson 1993). Gluscosamine and D-glucosamine hydrochloride were chosen as model compounds. Near-infrared spectroscopy is an appropriate method for the determination of OH and NH groups, where chitin/chitosan possesses both O–H and N–H groups. A multivariate regression method has been also employed to evaluate quantitatively the DA using NIR spectra (Vårum et al. 1995). A reference curve was constructed by plotting the DA estimated from NIR data versus the DA determined by ^{1}H NMR spectroscopy. A detailed description on the DA determination by NIR technique has been reviewed recently (Kasaai 2008).

12.3 DISCUSSION

12.3.1 General Aspects

Chitin naturally occurs associated with proteins, organic pigments, and minerals (Muzzarelli 1977, No 1995). Among them, proteins possess functional (amine and NH-C=O) groups similar to chitin/chitosan. The impurities and moisture create interference peaks, and change the positions

and intensities of some peaks in IR and NMR spectra of the polymers. Small differences in the compositions result in significant difference in NMR and IR spectra. An intense peak corresponds to OH group have been observed in IR and ^{1}H NMR spectra of chitin/chitosan. This is due to the hygroscopic nature of chitin/chitosan. Chitin/chitosan frequently associated with other polysaccharides, which make the evaluation of *N*-acetyl content by ^{1}H NMR, ^{13}C NMR, and IR more problematic and in some cases impossible. The solubility of chitin/chitosan in aqueous solution is limited due to the presence of strong hydrogen bonds and intermolecular interactions between macromolecule chains. The solubility of chitin/chitosan in aqueous acidic solution depends on the degree of crystallinity, degree of polymerization, degree of neutralization of amine groups, distribution of glucosamine and *N*-acetyl glucosamine residues in chitin/chitosan macromolecules, ionic strength of solvent, and pH and concentration of the polymer solutions. A detailed description of several parameters affecting on the chemical structure and the DA measurement have been appeared elsewhere (Kasaai 2008).

Broadening and overlapping frequently are also observed in spectroscopy, which induce difficulty to select a resolved absorption band. An accurate result is obtained when a sharp and well-resolved peak is chosen. Quantitative analysis by spectroscopy methods is comparison of responses with standards or references. This requires either the determination of an absorption band ratio or construction of a calibration curve by plotting absorption ratio versus the DA.

NMR or IR spectrum of an oligosaccharide or a polysaccharide is essentially a superposition of individual glycosyl residues, which are slightly modified because of the effect of linkages on the superimposed sample (van Halbeek 1996). Chito-oligosaccharides and small macromolecules result in clearer and more resolved NMR or IR spectra compared to the large macromolecules. Each unit residue of chitin and chitosan has six carbon and seven hydrogen atoms that are involved in CH linkages, and four hydrogen atoms in OH groups. Each of these nuclei or absorption bands in the unit residue has its own characteristics environment and thus its own chemical shifts or wave numbers.

Both β-(1-4) glucosidic linkages and *N*-acetyl groups are susceptible to acid or alkali hydrolysis. Generally, acid is used as a component of chitin/chitosan solvent in aqueous solution. If hydrolysis is occurred on β-(1-4) glucosidic linkage, results in a decrease in molecular weight of the polymers. If hydrolysis is taken place on *N*-acetyl groups, a decrease in the DA is obtained. The rate of degradation depends on the concentration of acid and temperature. Strong or concentrated acids are not appropriate solvents due to severe degradation of the polymers chains and *N*-acetyl groups. The degradation of *N*-acetyl groups increases with an increase in temperature. Dilute acid solution and room temperature are appropriate conditions for preparation of the sample solutions. The chemical structure of the polymer sample upon dissolution can be analyzed by IR or ^{1}H NMR spectroscopy. The change in chemical structure or viscosity of its solution is an indication of degradation process. Oxidative degradation of chitin/chitosan may also occur in presence of oxygen. Oxygen in the air promotes the oxidative degradation, especially when the temperature is high. If the oxidative degradation of the polymer occurs, the end unit residue should not be the same as the co-units of the original chitin/chitosan. This structural modification results in change in the solubility of the polymers. Alkali treatment of chitin as well as conversion of chitin into chitosan is generally accompanied by a chain scission of the backbone and result in shorter chains than the original chitin.

General difficulties in spectroscopy methods are: to choose an appropriate measuring signal; to choose an appropriate reference signal; and drawing a good baseline. There are two concepts, resolution and signal-to-noise ratio (S/N), which occur frequently in all types of spectroscopy (Graybeal 1988). Broadening and overlapping are observed in spectroscopy. These induce difficulty to select a resolved absorption band. An accurate result is obtained when a sharp and well-resolved peak is selected. Usually a signal-to-noise ratio of 2 or 3 is acceptable. One may measure the background noise of a blank sample to determine relative standard deviation (RSD) of the response. The limit of detection equals to the value of RSD multiplies by a factor 2 or 3. Generally, the limit of quantification equals the limit of detection multiplies by a factor of 5–10 (Lacroix 1995, Fischbacher 2000).

Chemical shifts or wave numbers are characteristic of particular molecular environments (Colquhoun and Goodfellow 1994). Chemical shift of particular resonance or wave number of particular absorption band varies by changing the distribution of two co-monomer units of chitin/chitosan, depending on the nature of the neighboring units (Schanzenbach and Peter 1997).

The chemical structure of the two polymers can be characterized by several techniques: Fourier transform infrared (FTIR); nuclear magnetic resonance (NMR); x-ray diffraction (XRD); transmission electron microscopy (TEM); scanning electron microscopy (SEM); mass spectroscopy (MS); ultraviolet spectrometry (UV); and electron scanning for chemical analysis (ESCA). The chemical structure of the two polymers can be analyzed by IR, NIR, or various types of NMR spectroscopy. Determining the structure of chitin and chitosan usually requires the application of combination of various methods. The combination of IR, NIR, and various techniques of NMR give ample information on the chemical structure. IR, NIR, and various types of NMR are less sensitive than that of other quantitative analysis such as UV, HPLC, GC, and MS.

A complete structural determination of chitin/chitosan, subsequent to its isolation and purification, involves the determination (the constituent of the two monosaccharides; the molecular size; the molecular size distribution; the linkage positions between the monomer units; their sequence and degree of heterogeneity; and any preferred conformation of the molecule(s) present). NMR spectroscopy can be used for structural characterization and conformational analysis of oligosaccharides in solution (van Halbeek 1996, Chaplin 2000). Conformational changes accompanied with metal-binding were examined by means of ^{13}C NMR spectroscopy (Saitô et al. 1987a,b).

The conformation of chitin/chitosan in solution changes as a function of temperature, solvent, pH, ionic strength, and concentration of counterions (Rinaudo et al. 1993). The conformation in solution may be completely different from that of in solid state. In solution, the flexibility of certain glycosidic linkages produces different conformations those co-exist in equilibrium (Imberty and Pérez 2000). Isolation, purification, and modifications are more likely to affect the structure and conformation of chitin/chitosan (Focher et al. 1992a,b). Two different monomer units in chitin/chitosan join by glycosidic linkages to form oligomers and polymers. They may be joined in random or block manner. Oligo- and polysaccharides of chitin and chitosan have a number of structures because of the various configurations possible for the two monomer units [2-acetamido-2-deoxy-D-glucose (*N*-acetyl glucosamine, GlcNAc) and 2-amino-2-deoxy-D-glucose (glucosamine, $GlcNH_2$)], with β-D-(1 → 4) glycoside linkages.

IR and NMR techniques compare with other analytical methods such as UV, HPLC, GC, and MS require a large quantity of sample or a long acquisition time to obtain a reasonable S/N ratio. This is due to low sensitivity of IR, NIR, and various types of NMR spectroscopy (Saalwächer and Ramamoorthy 2006). At least 10 nmol of chitin/chitosan is required to record NMR spectra. 1-D NMR requires between about 10 nmol (600 MHz) and 80 nmol (300 MHz) and 2-D NMR requires somewhat more (van Halbeek 1996, Chaplin 2000). Approximately 5, 300, and 200 mg of chitin/chitosan samples have been used to record ^{1}H NMR, ^{13}C NMR, and ^{15}N NMR spectra, respectively (Hirai et al. 1991, Ottøy et al. 1996, Yu et al. 1999). Measurements can be made with concentrations of 1–10 mg/mL and up to 100 mg/mL for IR and near-IR regions, respectively. These concentrations are required to produce sufficient absorption values. Generally, the amount of samples required for analysis depends on the sensitivity and limit of detection of the instrument. The instruments made with high and advanced technologies are more sensitive and required smaller amounts of samples for analysis.

12.3.2 NMR Spectroscopy

12.3.2.1 General Considerations

NMR spectroscopy has been used for qualitative and quantitative analysis. All characteristics of a signal (chemical shift, multiplicity, line-width, coupling constants, and relative intensity) contribute analytical information. Chemical shifts provide information on the chemical environment of the nuclei. The multiplicity gives important stereochemical information. Line width contributes

information on the resolving power of a spectrometer on a particular resonance. The magnitude of the coupling constant, *J*, between residual protons is used to identify the relative position of the substituent. The NMR spectra can be adequately described by the numeric value of the chemical shifts and coupling constants. Inadequate specimen preparation or incorrect instrumental and parameters adjustment may lead to poor resolution, decrease sensitivity, spectral artifacts, and erroneous data.

A comparison of a spectrum as a reference with that of an unknown specimen under investigation may be used to confirm the identity of a compound and to detect the presence of impurities that generate extraneous signals. Several special techniques (double resonance, chemical exchange, use of shift reagents, two-dimensional analysis, etc.) are available to simplify some of more complex spectra; to identify certain functional groups; and to determine coupling correlations.

The sensitivity and limit of detection are important parameters for quantitative analysis. NMR is a relatively low sensitive technique. The basic reason for the low sensitivity is the small difference in energy between the excited and the ground states (0.02 calories at 15–20 kilogauss field strength) results in a population difference between the two levels of only a few parts per million. Low sensitivity of ^{13}C NMR and ^{15}N NMR are associated with low natural abundance of ^{13}C and ^{15}N, especially when employed a low magnetic field strength spectrometer (Colquhoun and Goodfellow 1994, Bush 1996). In order to increase the sensitivity, it is necessary to use chitosan enriched with ^{13}C and ^{15}N. The low sensitivity of ^{13}C NMR and ^{15}N NMR spectroscopy leads to a long time accumulation for low concentration of the polymer. The application of artificial neutral network (ANN) technology increases the sensitivity by two or three orders of magnitude (van Halbeek 1996).

Another important aspect of the NMR technique, with a negative effect on the sensitivity, is the long lifetime of most nuclei in the excited state, which affects the design of the NMR analytical test, especially in pulsed repetitive experiments. Simultaneous acquisition of the entire spectrum instead of frequency swept can give sensitivity enhancement. Stronger magnetic fields and the ability to accumulate signals over long period of time greatly enhance the sensitivity of the method. The classical continuous wave (CW) instrument has low sensitivity and needs long analysis time. In pulse Fourier transform (FT) instrument, a single pulse of radio frequency energy is used to simultaneously activate all nuclei. The cross polarization and magic angle spinning have been used to obtain high resolution and to approach a line-narrow spectrum (Colquhoun and Goodfellow 1994, Braun et al. 1996, Ulrich 2000).

The intensity of a given signal is proportional to the number of nuclei contributing to the signal. This allows a quantitative determination of the relative amounts of two substances present in a sample, and the measurement can be made effectively by adding a carefully measured concentration of one of these substances followed by recording of the spectrum (Freeman 1988).

Peak asymmetries cause problems in measuring accurate chemical shifts values. The broadness, low signal-to-noise ratio and asymmetries make difficult the quantitative analysis. Reliable information can be obtained by lines fitting using Gaussian functions and take into consideration of full width at half-height (fwhh). These considerations reduce the error of the DA calculation specially arising from low value of the DA (Heux et al. 2000).

12.3.2.2 ^{1}H NMR

A typical spectrum of ^{1}H NMR spectroscopy is illustrated in Figure 12.2. The ^{1}H NMR spectrum of an oligosaccharide or a polysaccharide show that peaks at around 1.9–2.1 ppm represent the three *N*-acetyl protons of *N*-acetylglucosamine (GlcNAc), and peaks at 3.1–3.2 ppm represent a H-2 proton of glucosamine (GlcN) residues. The ring protons (H-3, 4, 5, 6, 6′) are considered to resonate at 3.6–4.0 ppm, and the peaks at 4.6 and 4.8 ppm were assigned to the H-1 protons of the GlcN and GlcNAc residues, respectively (van Halbeek 1996, Signini and Campana 1999, Lavertu et al. 2003). The nonanomeric protons ring-skeleton protons in a glycosyl residue have very similar electron densities and all of their signals appear between 3 and 4 ppm. Thus, the center of chitin/chitosan spectrum (between 3 and 4 ppm) is a crowded region. In the spectrum of an oligosaccharide, the signals of the

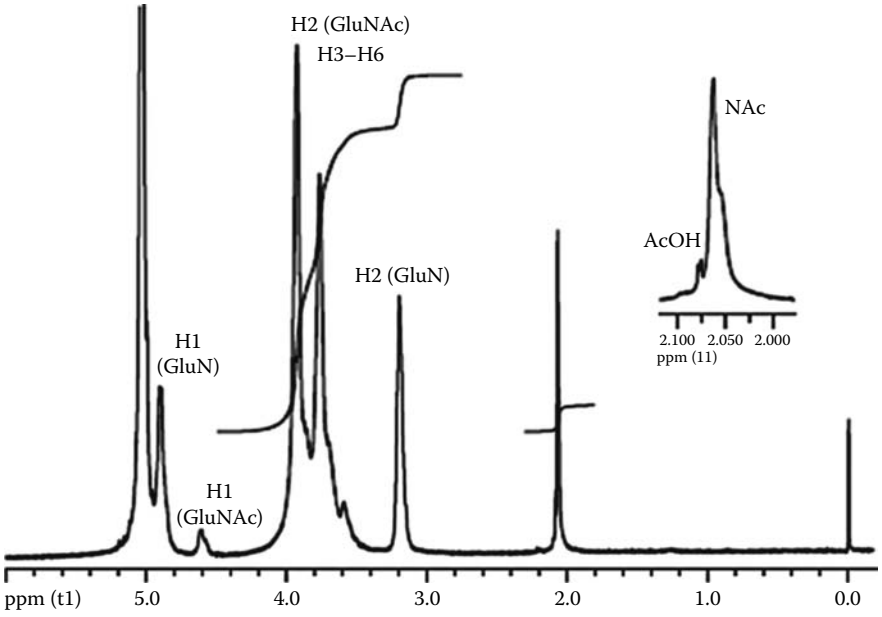

FIGURE 12.2 ^{1}H NMR spectrum of chitosan solution in 2% DCl/D_2O at 27°C using spectrometer (500 MHz). (From Fernandez-Megia, E. et al., *Carbohydr. Polym*., 61, 155, 2005. With permission.)

nonanomeric protons partially overlap; they constitute a broad envelope of signals in the middle of the spectrum having little fine structure and therefore almost no readily accessible structural information. These signals also overlapped with HOD signals at 4.05 ppm of the solvent (D_2O/CD_3COOD). The average values for chemical shifts of various protons are listed in Table 12.1 (Kasaai, 2010). The important structural signals for determination of the chemical structure are (1) the anomeric proton (H-1); (2) the proton attached to the carbon atoms in the direct vicinity of a linkage position; (3) the protons attached to deoxy carbon atoms; and (4) methyl protons of *N*-acetyl group (van Halbeek 1996). Anomeric protons (H-1) show signals at higher chemical shifts due to their neighboring glycosidic and ring oxygen [A proton in an equatorial position at a certain carbon atom in a pyranose ring has a chemical shift ~0.5 ppm higher than the corresponding axial proton attached to the same carbon. Therefore, D-residues involved in β-anomeric linkages (H-1 axial) has chemical shift about ~4.3–4.7 ppm]. Anomeric proton has been typically found doublets (van Halbeek 1996).

In ^{1}H NMR spectroscopy, chemical shifts and integration reveal the relative number of protons and the coupling patterns reveal information on the number of protons. Functional groups containing exchangeable protons bound to heteroatoms such as –OH or –NH_2 groups may be identified by taking advantage of the rapid exchange of these protons with D_2O. The resonance of these groups is replaced by the HDO singlet. This chemical exchange is an example of the effect of intermolecular and intramolecular rate processes on NMR spectra (van Halbeek 1996).

Chitosan as a polyelectrolyte with high molecular weight generates high solution viscosity. It is not possible to obtain clear and resolved spectrum using a viscous solution. In order to diminish the viscosity of solution and line width of ^{1}HNMR signals, ^{1}H NMR spectrum has been taken at temperatures between 70°C and 90°C (Hirai et al. 1991, Värum et al. 1991a, Tan et al. 1998, Heux et al. 2000). But the experimental time at high temperature is several times longer than that of room temperature. Fernandez-Megia et al. (2005) have demonstrated that the time of experiment significantly decreased (more than six times) by recording the spectrum at low temperature (27°C) compared to high temperature as high as 70°C. In addition, at 27°C, the residual HOD signal moves away from the integration area of the other protons of the polymers, and allows measurements of chitosans with high water content. Chemical shifts depend on temperature, however, this effect normally is small

TABLE 12.1
Chemical Shifts for Protons (in CD_3COOD/D_2O or DCl/D_2O), Carbons, and Nitrogens in Chitins and Chitosans Determined by ^1H NMR, ^{13}C NMR, and ^{15}N NMR Spectroscopy

Type of Nucleus	Position[a] (δ, ppm)
Proton	**Chemical Shift (δ, ppm)**
H_1 (H_1 of GluNAc)	4.62–4.85
H_1 (H_1 of $GluNH_2$)	4.85–4.97
H_2 (H_2 of $GluNH_2$)	3.18–3.24
H_2 (H_2 of GluNAc)	3.38–3.65
H_3 (H_3 of $GluNH_2$)	3.52–3.87
H_3 (H_3 of GluNAc)	3.52–3.65
H_3, H_4, H_5, H_6, H_6'	3.74–4.34
$H_{N–COCH3}$	1.95–2.09
CH_3COOH (AcOH)	2.09–2.11[b]
Carbon	**Chemical Shift (δ, ppm)**
C_1	102.7–105.7
C_2	55.2–57.6
C_3	73.1–75.7
C_4	80.9–85.7
C_5	73.1–75.7
C_6	59.6–60.8
$N–CH_3$ (C_7)	22.8–23.3
$N–C=O$ (C_8)	173.6–173.8
Nitrogen	**Chemical Shift (δ, ppm)**
NH_2	101–110
$NH–COCH_3$	0–10

Source: Kasaai, M.R. *Carbhydr. Polym.*, 79, 801, 2010. With permission.

[a] The lowest and highest values correspond to fully *N*-deacetylated chitosan and fully acetylated chitin, respectively.

[b] Acetic acid is produced by hydrolysis of *N*-acetyl residue of chitosan when the sample is kept at high temperature for a long time (For e.g., longer than 6 h at 90°C).

(Koenig 1992). Among various conditions proposed for determining the DA of chitosan by ^{1}HNMR (Hirai et al. 1991, Värum et al. 1991a, Rinaudo et al. 1992, Lavertu et al. 2003), the procedures proposed by Hirai et al. (1991) and Värum et al. (1991a) have been widely accepted.

Solid-state ^{1}H NMR spectroscopy results in higher resolution by working at high magnetic field strengths and applying high MAS speed (Ulrich 2000). One may apply solid-state ^{1}H NMR to investigate in chemical structures of chito-oligosaccharides and small macromolecules. Hydrogen possesses the highest magnetic moments compared to other nuclei present in chitin/chitosan. This gives it the greatest NMR sensitivity and stronger-dipolar coupling. Spin (I = 1/2) makes it well suited for structural investigations, and ^{1}H NMR is the most widely used nuclei in liquid state. In the solid state, the advantages of strong dipolar interactions, conveying long-range distance information, turns to a disadvantages when rapid spin diffusion occurs between nuclei in an extensively coupled network (Ulrich 2000).

12.3.2.3 ^{13}C NMR

^{13}C nucleus has a wide range of chemical shifts (0–250 ppm), which offers an excellent opportunity for chemical characterization of the different carbons. The large chemical shift dispersion of ^{13}C NMR spectra leads to well-resolved spectra (Bush 1996, Heux et al. 2000). There are essentially eight signals in spectrum of chitin/chitosan. These signals are attributed to the eight carbons of *N*-acetyl glucosamine residues (see Table 12.1). Each carbon atom has a particular molecular environment as well as chemical shift. Each signal occurs as singlet with the exception of carbons C_2 and $C_{C=O}$ which are doublets. These are due to the influence of the quadrupolar ^{14}N nucleus of the acetamide group (Tanner et al. 1990, Gail et al. 1991). The carbon atoms of pyranoyl residues are typically observed between 65 and 110 ppm. The anomeric carbon atoms reflect the configuration at the anomeric center. The methylene signals of monosaccharide (CH_2OH groups) are observed at ~60–70 ppm. A typical ^{13}C NMR spectrum of chitin/chitosan was shown in Figure 12.3. Carbon atoms of $-CH_2OH$ (C-6) group are clearly visible around 60 ppm. In D_2O solution refers to DSS, the anomeric signals are dispersed between 95 and 110 ppm and methyl carbon resonances are observed within 15–25 ppm (Bush 1996).

Two types of carbon atoms (CH_3 and C=O) were used to determine the DA. CH_3 signal is more appropriate than C=O signal, because a longer relaxation time for carbon nucleus of carbonyl group results in underestimated value for the DA (Saito et al. 1982, Raymond et al. 1993, Ottøy et al. 1996). Duarte et al. (2001b) determined the DA of chitin and chitosan samples using the procedure proposed by Raymond et al. (1993).The spectra of accurately weighed mixtures of chitin/chitosan and glycine were recorded at different times of contact, relaxation, and proton spin–lattice relaxation. The intensities of peaks for carbonyl group of glycine molecule and all carbon atoms of chitin/chitosan in the spectrum of the mixture allow estimation for amount of carbon in the mixtures. Ottøy et al. (1996) have determined the DA using both ^{13}C NMR and ^{1}H NMR spectroscopy. The DA of chitin/samples determined by ^{13}C NMR in the range of 20–55 was consistent with the DA values obtained from ^{1}H NMR.

12.3.2.4 ^{15}N NMR

A typical spectrum of ^{15}N NMR spectroscopy is illustrated in Figure 12.4. In the ^{15}N NMR spectrum of chitin/chitosan, there is only two peaks corresponding to acetamide ($NH–CO–CH_3$) and amine

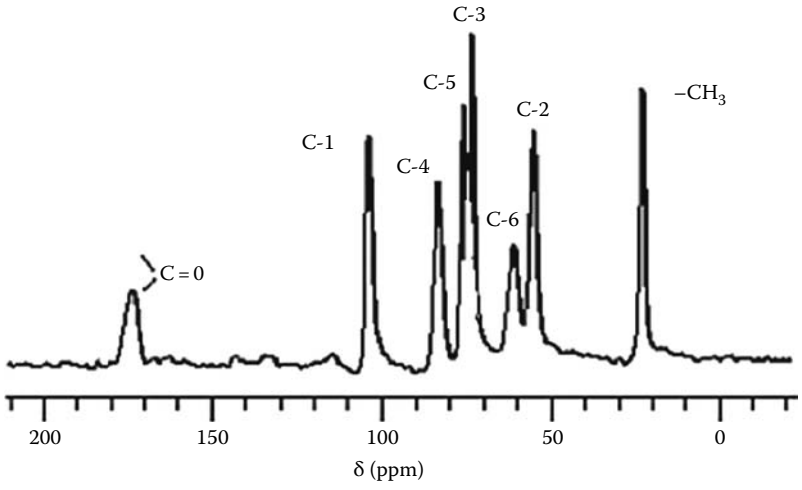

FIGURE 12.3 ^{13}C CP-MAS NMR spectra of chitin in solid state using magic angle of 4.5 kHz. The contact time and recycle delay δ is chemical shift with a unit of ppm were 1.8 ms and 13 s, respectively. Tetramethylsilane (TMS) was reference time. (From Guinesi, L.S. and Cavalheiro, E.T.G., *Thermochim. Acta*, 444, 128, 2006. With permission.)

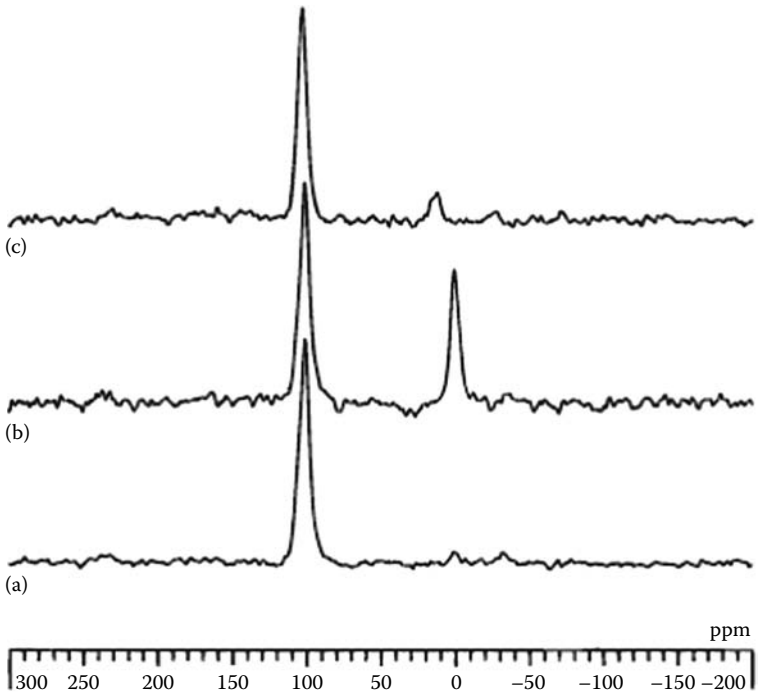

FIGURE 12.4 Solid-state ^{15}N CP-MAS NMR spectra of chitin samples: (a) the original chitin; (b) the sample after NaOH hydrolysis of the original chitin for 6 h; (c) the chitin sample obtained from a further treatment using 3 N HCl at boil for 1.5 h after NaOH hydrolysis for 7 h. (From Yu, G. et al., *Macromolecules,* 32, 518, 1999. With permission.)

(NH_2) groups and the two peaks are far from each other and well-separated (Yu et al. 1999, Heux et al. 2000, Brugnerotto et al. 2001b). The chemical shift for nitrogen of *N*-acetyl group reported to be between 101 and 110 and smaller than 10 ppm for the amine groups depending on their neighbor environments. The difference in the chemical shifts reported by the two research groups could be due to the chitins having different the DA values. Yu et al. (1999) determined the DA% in the range (60–95) by ^{13}C NMR and ^{15}N NMR spectroscopy, whereas Heux et al. (2000) determined the DA% in the range (0–100). The chemical shifts for different *N* nuclei in chitin/chitosan are given in Table 12.1. The lowest value of the chemical shift is corresponded to fully N-deacetylated chitosan and the highest value is attributed to fully acetylated chitin.

In the range of $0 < DA < 10$, ^{15}N NMR is less sensitive than ^{13}C NMR spectroscopy. One cannot expect to detect the DA level lower than 10% with ^{15}N NMR technique, when line broadening effects are observed. Linewidth or full width at half-height (fwhh) in ^{15}N NMR technique gives a good indication of crystallinity of the sample. ^{15}N NMR technique can be employed to evaluate the DA for chitin in association with (1 → 3)-β-D-glucans or structural polysaccharides of fungi (Heux et al. 2000). The results of ^{15}N NMR spectroscopy were consistent with the results of ^{13}C NMR in the range (60–95) (Yu et al. 1999).

12.3.3 IR and Near-IR Spectroscopy

12.3.3.1 IR

The absorption bands and their corresponding wave numbers are listed in Table 12.2. The important spectral regions at 3000–3300 cm^{-1} are generally for the elucidation of the free or the interacting hydroxyl (–OH) groups; at 2850–2950 cm^{-1} for the alkyl ($–CH_2–$) chains; and at 1650–1750 cm^{-1},

TABLE 12.2
Absorption Bands and Their Corresponding Wave Numbers for the Region of IR

Absorption Band (IR)	Wave Number (cm^{-1})
O–H stretching	3445–3455
Intermolecular OH…3 and CH_2OH hydrogen bonds	3380–3420
Intermolecular $C(2_1)NH….O{=}C(7_3)$ hydrogen bonds	3250–3260
Intermolecular $C(6_1)OH….HOC\ (6_2)$ hydrogen bonds	3140–3150
N–H stretching	3270–3290
C–H stretching	2870–2900
C=O stretching	1795–1820
C=O–NH–CH_3 stretching (amide I band, α-chitin, β-chitin, and partially deacetylated chitosan)	1620–1660
N–H bending of NH_2	1620–1630
NH_2 band (chitosan)	1590–1610
Amide II band	1550–1565
Quaternary ammonium acetate(chitosan hyroacetate)	1550–1570
NH_3^+	1510–1520
CH_2 bending	1420–1430
C–H bending and C–CH_3 deformation mode	1375–1382
Amide III band	1310–1320
C–O–C stretching	1020–1030
C–O–C stretching	1070–1075
C–O–C bridge (gluosidic linkage)	890–900
Glucose linkage/C–O–C bridge [asymmetric (antisymmetric) stretching]	1155–1165
C–O bending	715–720

the bands represent the carbonyl groups (–C=O) and the amides bands (–C–N–). Figure 12.5 shows IR spectrum of chitin. Several absorption ratios have been proposed to determine the DA. Most of them can be used for a limited range of the DA. This is due to some peaks appeared with an increase in acetylation or deacetylation, where other peaks disappeared (Kasaai 2008). The determination of the DA by IR spectroscopy is a flexible method, because several measuring bands may be chosen. There is no absorption ratio allowing the determination of DA for the entire range of the DA with a good accuracy. The validity of estimated DA for unknown chitin and chitosan samples was examined from the knowledge of the DA determined by ^{1}H NMR or another validated method using standard chitin/chitosan samples. The range of the DA for standard samples must cover the range of the DA for unknown samples (Kasaai 2008).

For a quantitative analysis, it is necessary to create a calibration curve using several chitin/chitosan samples having a known DA. The procedures of Duarte et al. (2002) and Vårum et al. (1995) can be used for quantitative evaluation of the DA. The advantage of the procedure of Duarte et al. (2002) over other procedures is that several absorption ratios were used to determine the DA using only one spectrum. The statistical evaluation of the data resulting from the combination of all the absorption ratios yields in a DA with a higher accuracy compared to applying only one ratio. Brugnerotto et al. (2001b) compared the results of DA obtained from the absorption ratios of A_{1320}/A_{1420} and A_{1650}/A_{3450}. They obtained superior agreement for the ratio of A_{1320}/A_{1420}. The reference data for comparison of the two ratios was the DA those obtained from ^{1}H NMR and ^{13}C NMR spectroscopy.

In most of the results reported by research groups, linear relationships for the plots of absorption ratios versus the DA (for a limited or whole range of DA) were observed. The regression formula allows one to predict the DA from the absorption ratio data. The relationship was based on the

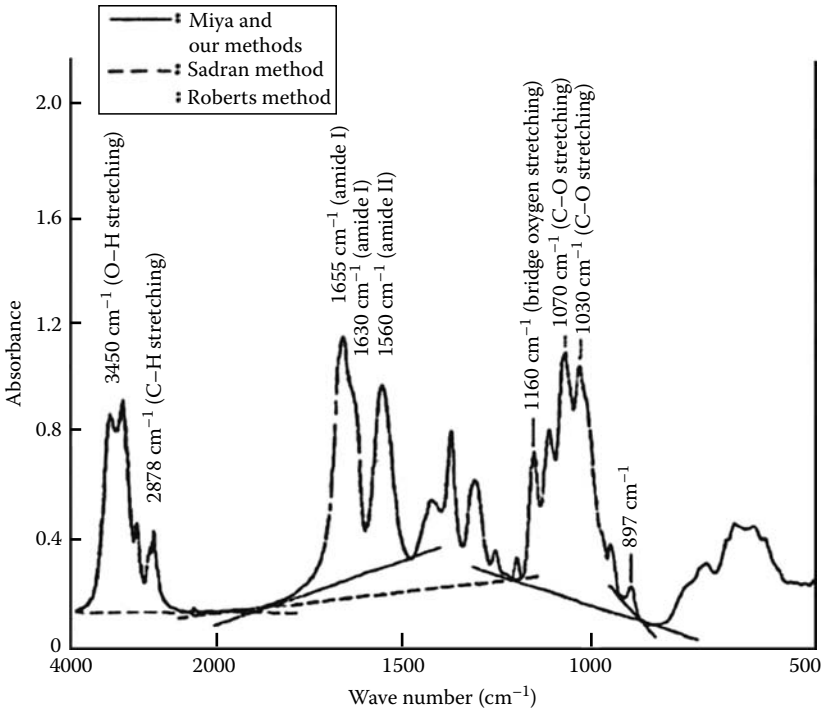

FIGURE 12.5 IR spectrum of chitin and appropriate baselines. (From Shigemasa, Y. et al., *Int. J. Biol. Macromol.*, 18, 237, 1996. With permission.)

assumption that the relationship between variables (absorption ratio versus DA) is linear. One needs to calculate regression coefficients A and B to predict the DA:

$$Y = A \cdot \mathrm{DA} + B \tag{12.7}$$

One can determine the slope (A) and intercept (B) from the linear plot. A detailed description has been published elsewhere (Kasaai 2008).

If a good baseline is selected and IR spectroscopy results in a highly resolved spectrum, it is a good initial data for a quantitative analysis. The line width gives a good indication of the crystallinity of samples. Crystalline samples result in higher resolved spectra than those of amorphous samples, whereas amorphous samples produce broad absorption bands (Gail et al. 1991, Brugnerotto et al. 2001b). For a quantitative analysis with a high accuracy, the chief limitations of IR method are requirement of sophisticated procedures such as (1) statistical evaluation of various absorption ratios, (2) removal of interferences from the samples, and (3) requirement of long time for the DA calculation.

12.3.3.2 Near-IR

Near-infrared spectroscopy is an appropriate method for determination of OH and NH groups. Chitin/chitosan possesses the two latter groups. NIR spectroscopy offers a powerful alternative to gain information regarding hydrogen bonds. The energies of hydrogen bonds are weak (20–50 kJ/mol) in comparison to covalent bonds (order of 400 kJ/mol). The hydrogen bond interactions are large enough to produce appreciable frequency and intensity changes in the vibrational spectra (Eschenauer et al. 1992). The shift in frequency has been correlated with the energies of hydrogen bonds formation. Thus, NIR can be used to distinguish different morphology (α- and β-chitin). The (–OH, –NH, and $–NH_2$) groups act as proton-donating groups and the (–C=O, –OH, –NH,

and $-NH_2$, –O–) groups act as hydrogen- accepting groups in the formation of hydrogen bonds in chitin and chitosan.

Rathke and Hudson (1993) proposed near infrared spectroscopy as an accurate method to determine DA. They have chosen different absorption bands in the range of 4000–9090 cm^{-1} to determine the DA% ≤ 60. Monomer sugars, *N*-acetyl D-glucosamine and D-glucosamine hydrochloride, have been used as model compounds. The absorption ratio of A_{7669}/A_{7474} and A_{6039}/A_{5342} were chosen as the best ratios for the model compounds and chitin/chitosan samples, respectively. The wavelength at 7669 cm^{-1} was chosen, because it had the highest correlation with the DA of monomer mixtures. The intensity of the band at 7474 cm^{-1} was invariant to different samples prepared from mixtures of the monomers. The calibration curve was constructed by plotting: the absorption ratio of A_{7669}/A_{7474} versus the mole percent of *N*-acetyl D-glucosamine. Twenty-one chitin/chitosan samples including the monomers and mixtures of monomers were employed to construct a calibration curve. The second calibration curve was constructed by plotting the ratio of A_{6039}/A_{5342} versus DA% in the range of 8%–22% using five chitosan samples. A detailed description on the calibration curves and DA determination can be found in the original (Rathke and Hudson 1993) or the review article (Kasaai 2008). There was a shift in the NIR spectra of chitosan around 4892 cm^{-1} compared to the monomer of *N*-acetyl-D-glucosamine and glucosamine (Rathke and Hudson 1993). This is due to the presence of hydrogen bonds in the polymeric backbone of chitosan compared to its monomer units.

Vårum et al. (1995) analyzed the data obtained from NIR spectra (4000–9090 cm^{-1}) using the multivariate calibration method. A calibration curve of DA determined from NIR spectra versus the DA determined from ^1H NMR spectroscopy was constructed in order to predict the DA and to optimize the accuracy. The correlation coefficient and root mean square for the plot (DA% = 0–60) were 0.985% and 3%, respectively.

12.3.4 Determination of Morphology and Structural Characterization of Chitin/Chitosan by NMR and IR Techniques

Chitin has two principal polymorphous forms (α, β) (Muzzarelli 1977, Blackwell et al. 1978, Brown 1982, Blackwell 1988). γ-Chitin has been also reported (Takai et al. 1989). However, the latter form actually does not exist. It is simply a mixture of α- and β-chitins. Polymorphism may be occasionally responsible for a difference in the NMR and IR spectra of a given compound in the solid state. Frequently, small differences in structure result in difference in NMR and IR spectra (Saito et al. 1997). α-Chitin is the dominant and the most abundant polymorphous form found in crustaceans shells, insects, and fungi. β-Chitin is found in squid and marine diatoms (Muzzarelli 1977, Blackwell et al. 1978, Gorovoj and Burdukova 1996). α-Chitin is more crystalline, more stable, less soluble, and less hygroscopic than that of β-chitin. It forms an antiparallel structure and all of hydroxyl groups are involved in hydrogen bonds, whereas β-chitin forms a parallel structure and it consists an array of poly-*N*-acetyl-D-glucosamine chains (Gardner and Blackwell 1975, Minke and Blackwell 1978, Takai et al. 1989, Kurita et al. 1991, Lamarque et al. 2004). The morphology of chitin/chitosan can be distinguished by NMR and IR spectroscopy and XRD. These techniques provide information on crystallinity of a polymer sample (Focher et al. 1992a,b). IR and NMR spectroscopy measure short-range crystallinity and XRD measure long-range crystallinity (Koenig 1992). Short-range order describes intramolecular phenomenon. The short-range intramolecular order is a necessary condition for the occurrence of long-range intermolecular order, but the short-range order can exist without the presence of the long-range order.

The degree of crystallinity has been determined from the ratio of A_{1380}/A_{2920} (Focher et al. 1990, Prashanth et al. 2002). The sharp band at 1380 cm^{-1} corresponds to a symmetrical deformation of $C-CH_3$. It is probably superimposed with CH bending around 1380 cm^{-1} (Duarte et al. 2001a,b, 2002). Configuration of C_3 and C_5 carbon atoms in α- and β-chitin are different. This is due to differences in hydrogen bonding forces. In ^{13}C CP/MAS NMR spectra of α-chitin, two signals were appeared around 73 and 75 ppm. These are sharply resolved and are assigned to C_3 and C_5, whereas

in *N*-acetylated β-chitin a relative broad and single peak around 74 ppm corresponds to C_5 and C_3 are appeared. This difference is probably due to a different configuration of C_5 and C_3 by hydrogen bonding (Takai et al. 1989, Kim et al. 1996). The ^{13}C NMR spectrum of α-chitin has been changed after deacetylation of chitin by NaOH (followed by washing, drying, and annealing) into chitosan. The changes were also observed in chemical shifts of C_1 and C_4. The difference is based on the conformational change. This result confirms that different molecular and crystal structure exists between two forms of chitosans (form I and form II) (Takai et al. 1989). However, three polymorphs of chitosan [tendon-chitosan from crab shell; L-2 from shrimp shell and annealed in the presence of water from crab shell] have been already reported as obtained by XRD data (Saitô et al. 1998). The two forms out of three correspond to changes in C-1 and C-4 signals during splitting. The third form has been formed by dehydration of water molecules those loosely bounded during either annealing or complex formation with transition metal ions (Saitô et al. 1998).

Chemical modification such as decomposition, acetylation, de-acetylation, fragmentation, or depolymerization processes results in changes in NMR and IR spectra by creation some new peaks, increasing/decreasing of the intensity or shifting the position of some peaks in NMR and IR spectra of chitin/chitosan samples. The formation of *N*-acetyl group in the process of N-acetylation leads to a significant variation of the chemical shift and absorption bands in IR and NMR spectroscopy. Upon deacetylation, a decrease in proton spin–lattice relaxation and cross-polarization relaxation times were observed. This is probably due to structural changes causes by deacetylation, such as loss of crystallinity through the breaking of hydrogen bonds (Värum et al. 1991a, Raymond et al. 1993).

IR technique has been used for qualitative analysis via comparison evaluation of the spectra of the polymer samples obtained from modification with the original one. Chitin/chitosan samples obtained from de-acetylation or acetylation process may be used for qualitative evaluation of DA and other structural modifications (Sannan et al. 1976, Domard and Rinaudo 1983, Focher et al. 1990, Kim et al. 1996, Knaul et al. 1998). The samples may be obtained from fragmentation or depolymerization process (Focher et al. 1992a, Kasaai et al. 1999).The effects of modifications on the chemical structures and the DA can be found elsewhere (Sannan et al. 1976, Domard and Rinaudo 1983, Focher et al. 1990, 1992a,b, Kim et al. 1996, Saito et al. 1997, Knaul et al. 1998, Kasaai et al. 1999).

12.4 COMPARISON OF VARIOUS TYPES OF NMR AND IR SPECTROSCOPY FOR STRUCTURAL CHARACTERIZATION AND THE DEGREE OF N-ACETYLATION DETERMINATION

The DA range, advantages, and disadvantages of various types of NMR and IR techniques to determine the chemical structure and the DA for chitin and chitosan are given in Table 12.3. This study enables one to compare various spectroscopy methods to determine the chemical structure and the DA of chitin/chitosan. IR technique can be used mainly for qualitative analysis and comparison studies. For quantitative analysis with a high accuracy, the chief limitations of the IR method are that (1) sophisticated procedures are required, such as statistical evaluation of various absorption ratios; removal of interferences from the sample; and the long-term and time-consuming process of calculation. It can be used for quantitative analysis of crystalline samples, since crystalline samples created sharper signals and higher resolution compared to amorphous samples. The quantitative analysis for the DA of amorphous samples is more difficult than that of crystalline ones, because the former ones produce broad absorption bands. ^1H NMR spectroscopy has been employed to verify the validity of several techniques (Shigemasa et al. 1996a,b, Brugnerotto et al. 2001a,b) for the DA determination. The validity of IR technique has been examined by ^1H NMR technique. ^1H NMR has been employed as standard method by American Standard Test Method (ASTM) organization to determine the DA for chitosan (ASTM 2003). The method has been published as the test F2260-03 in edition 2003. Among various NMR techniques, ^1H NMR results in more reliable data than that of ^{13}C NMR, ^{15}N NMR, and

TABLE 12.3
Different Methods for Structural Characterization and the DA Determination, Their Corresponding DA Ranges, Their Performances and Limitations

Method	DA Range	Performances	Limitations
^{1}H NMR	Liquid ^{1}H NMR is applicable for soluble chitin/chitosan	^{1}H NMR technique is more precise and more sensitive than other methods and result in more accurate data in comparison to other methods; some information can be obtained on the sequence and type of copolymers (random and block).	Applicable only for soluble chitin/chitosan samples; requires sample preparation; proteins, organic pigments and humidity are interferences; applicable for a limited range of the DA, where the sample is soluble in the solvent.
^{13}C NMR	0–100	The solid state ^{13}C NMR is applicable for soluble, insoluble structural analysis of chitin/chitosan samples; it can be used for entire range of the DA determination; no need to prepare and to dry the sample; some information on chemical structure, sequence of co-monomer units and type of copolymers (random and block) can be obtained from the spectra of chitin/chitosan samples; the more sensitive instrument generally results in the higher precision; resolution, limit of detection; and accuracy of results are improved using cross polarization and strong magnetic fields.	Low resolution and low sensitivity; the impurities of chitin/chitosan (moisture, protein, pigments, and metal ions) create interference peaks; ^{13}CNMR instrument is not widely available in many laboratories; the limit of detection is more than 5%.
^{15}N NMR	0–100	All of the advantages points described in ^{13}C NMR are valid for ^{15}N NMR; appropriate method for a composite or blend of chitin/chitosan with other polysaccharides; other polysaccharides are not interferences.	All of the disadvantages points described in ^{13}C NMR are valid for ^{15}N NMR; possible errors if the sample contains proteins as impurities.
IR	Varies with different ratios	Easy-to use-apparatus; it can be used for qualitative, comparative evaluation, and routine structural and DA analysis; more appropriate for the DA determination of crystalline samples; several possibilities as well as absorption ratios exist to choose absorption band ratios and determine the DA. It is more flexible than the other ones.	Drawing baseline is a general difficulty; humidity and impurities are interferences; and IR method usually requires carefully selected chitin/chitosan reference samples (with certain DA values).
Near-IR	Soluble samples	Accurate results for DA ≤ 60	Drawing baseline is a general difficulty; and NIR method usually requires carefully selected chitin/chitosan reference samples (with certain DA values).

IR techniques. The combination of proton, carbon, and nitrogen data gives significant information on the structure of the unknown sample (Blackwood and Chaplin 2000). The combination of three techniques of NMR would yield the determination of the DA in entire range for chitin/chitosan, their some derivatives and composites. The results of solid-state ^{13}C CP-MAS, and ^{15}N CP-MAS were in good agreement in entire range of DA (Heux et al. 2000). The authors have compared results of the three NMR techniques and they concluded that the three methods were in good agreement.

The detection limits for determination of the DA using ^{13}C NMR and ^{15}N NMR spectroscopy reported to be 10%. ^{13}C NMR and ^{15}N NMR techniques are not appropriate techniques for chitin/chitosan having low DA values. These two NMR techniques resulted in underestimates values for low DA values (Domard 1987, Raymond et al. 1993, Heux et al. 2000). The margin of error for the DA obtained by ^{1}H NMR spectroscopy reported to be around 5% (Hirai et al. 1991, Värum et al. 1991a, Rinaudo et al. 1992, Kasaai et al. 1999, Heux et al. 2000). ^{13}C NMR and ^{15}N NMR can be used for entire range of the DA. Solid-state NMR (^{13}C and ^{15}N) spectroscopy has been used to determine the DA of insoluble samples such as cross-linked and block copolymers. CP/MAS, ^{13}C NMR, and CP/MAS ^{15}N NMR do not need sample preparation for dissolution and are appropriate methods for insoluble chitin/chitosan samples even the samples are associated with impurities and humidity (Ottøy et al. 1996).

^{15}N NMR is less sensitive than ^{13}C NMR. ^{1}H NMR is the most sensitive method. Among various NMR and IR techniques, ^{1}H NMR spectroscopy can be considered as a precise and reliable one because it is the most sensitive and precise method and results in the least variation of experimental results as well as the most accurate data. The signal resolution of ^{1}H NMR is much better than those of ^{13}C NMR and ^{15}N NMR. The resolution of solid-state NMR is generally much worse than that of liquid (Colquhoun and Goodfellow 1994). This is because the orientation in solids is a very slow process and spin lattice relaxation time (T_1) values tend to be very long. Thus, little information can be extracted from solid-state NMR spectra. Cross-polarization and magic-angle-spinning devices are widely used to obtain high-resolution spectra (Colquhoun and Goodfellow 1994).

The ^{13}C NMR and ^{15}N NMR spectra of chitin/chitosan generally show much better chemical shift dispersion than do the ^{1}H NMR spectra. Interpretation of a ^{13}C NMR spectrum and the DA determination are not difficult, because ^{13}C nucleus has a wide range of chemical shifts (0–250 ppm); carbonyl and methyl groups are well resolved and different carbon atoms having different chemical shifts are well separated. The interpretation of ^{15}N NMR spectrum and evaluation of the DA measurement is simple, since it gives only two signals. Among polysaccharides, the *N* nucleus is only present in chitin and chitosan, thus the evaluation of DA in the two polymers associated with other polysaccharides using ^{15}N NMR spectroscopy is possible without any purification process.

^{1}H NMR and ^{13}C NMR spectroscopy may also provide information on the sequential distribution of the *N*-acetyl glucosamine and glucosamine residues in oligomers and polymers of chitin and chitosan (Värum et al. 1991a,b, Schanzenbach and Peter 1997, Brugnerotto et al. 2001b). Värum et al. (1991a) reported that GlcNAc and GlcN residues are distributed randomly in chitosan prepared under homogenous and heterogeneous conditions. ^{1}H NMR and ^{13}C NMR yielded in a slightly higher block wise distribution under heterogeneous *N*-acetylation conditions (Värum et al. 1991a,b). Various types of NMR and IR spectroscopy give information on morphology of chitin and chitosan.

12.5 CONCLUSIONS

In this chapter, the reported information on different NMR, IR, and NIR spectroscopy techniques on structural characterization and the DA determination of oligomers and polymers of chitin and chitosan have been described. The following conclusions were made from this study: (1) NMR and IR are nondestructive and noninvasive methods; (2) impurities (minerals, proteins, and pigments) and moisture induce interferences and difficulties to obtain accurate results. To achieve more accurate results, care must be taken to identify the peaks correspond to the impurities; (3) IR and NMR

have been used for qualitative analysis, comparison evaluation, and elucidation of the chemical structure and the DA determination. The samples were obtained from depolymerization or acetylation/deacetylation process. In this way, the spectra of depolymerized or de-acetylated/acetylated samples are compared with the original one in order to evaluate any structural modification that may occur during the processes; (4) the change in the distribution of *N*-acetyl and amine groups in partially deacetylated chitin or partially acetylated chitosans has a significant effect on NMR and IR spectra. Depending on the nature of neighboring units, the chemical shifts of particular resonances or wave numbers of particular absorption bands may vary due to conformational and configuration effects; (5) the DA of chitin/chitosan by various types of NMR (^{1}H NMR, ^{13}C NMR, and ^{15}N NMR) and IR (IR and NIR) have been determined as described in the following: determination of the ratio of A_p/A_R: where A_p is height or area of the probe signal; and A_R is the height or area of reference signal. The DA of unknown samples was estimated by comparing the values of A_p/A_R with a calibration curve. The calibration cure was constructed by plotting the ratio of A_p/A_R versus their corresponding DA values; (6) the morphology of chitin (α, β) and chitosan can be distinguished or compared by IR and NMR spectroscopy; and (7) ^{1}H NMR and ^{13}C NMR spectroscopy may also provide information on the sequential distribution of the *N*-acetyl glucosamine and glucosamine residues.

The accuracy of a method can be evaluated by comparison of the results of a desirable method with results of validate/precise method. Among various NMR and IR techniques, ^{1}H NMR spectroscopy can be considered as a precise and reliable one. This is due to the fact that it is the most sensitive and precise method and results in the least variation of experimental results as well as the most accurate data. It is desirable to take into consideration the following major parameters for quantitative analysis: time of measurement; precision of method; and accuracy of results. In this way, ^{1}H NMR is the best one for quantitative evaluation.

12.6 FUTURE WORK AND PERSPECTIVES

There is no unique method that can be obtained enough information on the chemical structure of polysaccharides. One should employ a combination of several analytical methods to gain significant information. NMR spectroscopy in combination with FT-IR, XRD, and chromatographic methods are the best means to determine the chemical structures for oligosaccharides and polysaccharides. NMR spectroscopy is the most powerful technique for the accomplishment of this task, from which useful information such as linkage position, sequence, size of macromolecules, and conformation can be obtained. The use of NMR spectroscopy in the determination/estimation of sequence and size of oligosaccharides and polysaccharides, as a branch area of research, are still in its infancy. The nuclei of interest in NMR studies for chitin and chitosan are ^{1}H, ^{13}C, ^{17}O, and ^{15}N. With development of multidimensional techniques such as two-dimensional (proton and carbon) and three dimensional (proton, carbon and nitrogen) and powerful (700 MHz) instrumentation, significant information can be achieved. No information is available on ^{17}O NMR of chitin/chitosan. ^{17}O NMR spectroscopy can be used to determine chemical structure of chitin/chitosan and evaluate the DA.

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Part III

Structural Modifications of Chitin and Chitosan Derivatives

13 Chemical Modifications of Chitosan Intended for Biomedical Applications

Mani Prabaharan and Ashutosh Tiwari

CONTENTS

13.1 INTRODUCTION

Chitosan, α (1-4) 2-amino 2-deoxy β-D glucan, possess structural characteristics similar to glycosaminoglycans. This polycationic biopolymer is generally obtained by alkaline deacetylation of chitin, which is the main component of the exoskeleton of crustaceans, such as shrimps (Muzzarelli 1973). Chitosan displays interesting properties such as biocompatibility and biodegradability (Ravikumar 2001), and its degradation products are nontoxic, nonimmunogenic, and noncarcinogenic (Bersch et al. 1995). Moreover, chitosan is metabolized by certain human enzymes, especially lysozyme, and is considered as biodegradable (Muzzarelli 1997). The physicochemical and biological properties of chitosan led to the recognition of this polymer as a promising material for biomedical applications. However, chitosan can only be soluble in a few dilute acid solutions, which limits its wide applications. Recently, there has been a growing interest in the chemical modification of chitosan to improve its solubility and widen its applications (Sashiwa and Shigemasa 1999). Although the chemical modification of chitosan modifies its properties, it is possible to maintain some interesting characteristics such as mucoadhesivity, biocompatibility, and biodegradability (Jayakumar et al. 2005). The aim of this chapter

is to review the recent developments on the chemically modified chitosan derivatives that are specially designed for drug/gene delivery, tissue engineering, and wound-healing applications.

13.2 IN DRUG DELIVERY

The chemical modification of chitosan is a powerful tool to control the interaction of the polymer with drugs, enhance the load capability, and tailor the release profile of the drug carriers. Chemically modified chitosan improves its bulk properties for the preparation of sustained drug release systems. Many studies in this context have been reported in the literature.

13.2.1 Thiolated Chitosan Derivatives

The modification of chitosan with 2-iminothiolane was prepared in order to improve the properties of chitosan as excipient in drug delivery systems, as shown in Figure 13.1 (Schnurch et al. 2003). Chitosan–2-iminothiolane showed excellent in situ gelling properties due to the formation of disulfide bonds based on an oxidation process of the immobilized thiol groups under physiological conditions. In addition, the mucoadhesive and controlled drug-releasing properties were also strongly improved by the covalent attachment of thiol groups on chitosan.

Roldo et al. (2004) studied the influence of the molecular mass and the amount of immobilized thiol groups on the mucoadhesive properties of chitosan–4-thiobutylamidine conjugates. This study reported that the mucoadhesive properties are increased when increasing the level of 4-thiobutylamidine substructures immobilized on the polymer. In addition, thiolated chitosan of medium molecular weight was identified as comparatively the most mucoadhesive polymer. Recently, Schnurch et al. (2004) also reported the permeation enhancing effect of chitosan–4-thiobutylamidine conjugate in combination with the permeation mediator glutathione. Release studies of this conjugate showed a threefold higher permeation-enhancing effect of the chitosan–4-thiobutylamidine conjugate/glutathione system in comparison to unmodified chitosan. Similarly, mucoadhesive properties of chitosan covalently attached with thioglycolic acid were studied by Kast and Schnurch (2001). The results showed that chitosan–thioglycolic acid conjugates exhibiting an up to 10 times improved mucoadhesion.

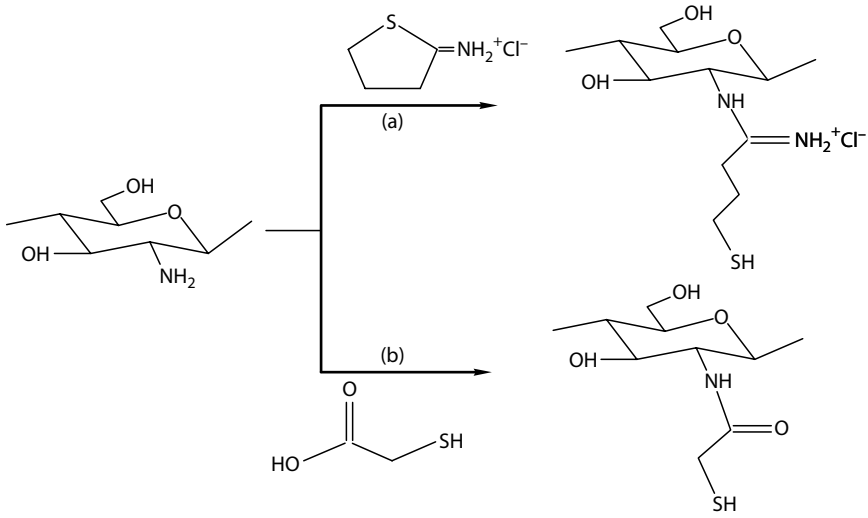


FIGURE 13.1 Synthetic pathway of (a) chitosan–4-thio butylamidine conjugate, (b) chitosan–thioglycolic acid conjugate. (From Schnurch, A.B. et al., *Int. J. Pharm.*, 260, 229, 2003.)

13.2.2 Amphiphilic Chitosan Derivatives

Colloidal systems have found numerous applications as promising delivery vehicles for therapeutics due to their low toxic side effects and enhanced therapeutic effects. The introduction of an alkyl chain onto chemically modified chitosan offers the presence of both hydrophobic and hydrophilic branches in its structure. A methodology for the preparation of *N*-lauryl-*N*-methylene phosphonic chitosan has been developed by Ramos et al. (2003) on the water soluble *N*-methylene phosphonic chitosan. The presence of alkyl groups in *N*-lauryl-*N*-methylene phosphonic chitosan seems to weaken the hydrogen bond and provides good solubility in organic solvents. As a result of the amphiphilic properties, like surface activity typical for surfactants, this derivative opens new perspectives in pharmaceutical field. *N*-acylation of chitosan with various fatty acid (C_6–C_{16}) chlorides increased its hydrophobic character and made important changes in its structural features. Tien et al. (2003) described the *N*-acylation of chitosan with fatty acyl chlorides to introduce hydrophobicity for use as matrix for drug delivery (Figure 13.2). Drug dissolution kinetics showed longer release times for higher degrees of functionalization, suggesting palmitoyl chitosan excipients as interesting candidates for oral and subdermal pharmaceutical applications. Moreover, it has been reported that polymeric micelle is better than other carriers for use as passive targeting carrier of anticancer drugs. *N*-lauryl carboxymethyl chitosan with both hydrophobic and hydrophilic groups was studied by Miwa et al. (1998) in connection with delivery of taxol to cancerous tissues.

The conjugation of lipid groups into chitosan molecule can create an amphiphilic self-aggregate molecule, which can be used as a drug delivery system. Uchegbu et al. (2001) have synthesized palmitoyl glycol chitosan (GCP) by reacting glycol chitosan and sodium bicarbonate with palmitic acid *N*-hydroxysuccinimide as an erodible controlled release system for the delivery of hydrophilic macromolecules. Prabaharan et al. (2007) prepared amphiphilic carboxymethyl chitosan (CMC)-*g*-phosphatidylethanolamine (PEA) as shown in Figure 13.3. The encapsulation efficiencies of ketoprofen as a model drug by sodium tripolyphosphate cross-linked CMC-*g*-PEA beads were found to be more than 68%. The amount of the drug release was much higher in acidic solution than in basic solution due to the swelling properties of the matrix at acidic pH.

13.2.3 Cyclodextrin-Grafted Chitosan

Cyclodextrins (CDs) have gained prominence in recent years because their hydrophobic cavity is capable of binding aromatic and other small organic molecules, and therefore provides ideal binding sites. CD-linked chitosan is interesting for the viewpoint of biomedical applications. Tojima et al. (1998) prepared α-CD-linked chitosan using 2-*O*-formylmethyl-α-CD beads by reductive

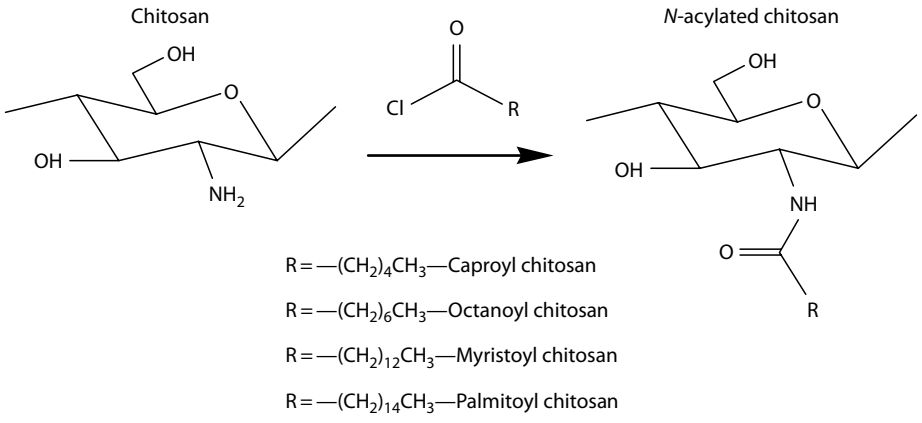


FIGURE 13.2 Chitosan derivatization with fatty acyl chlorides.

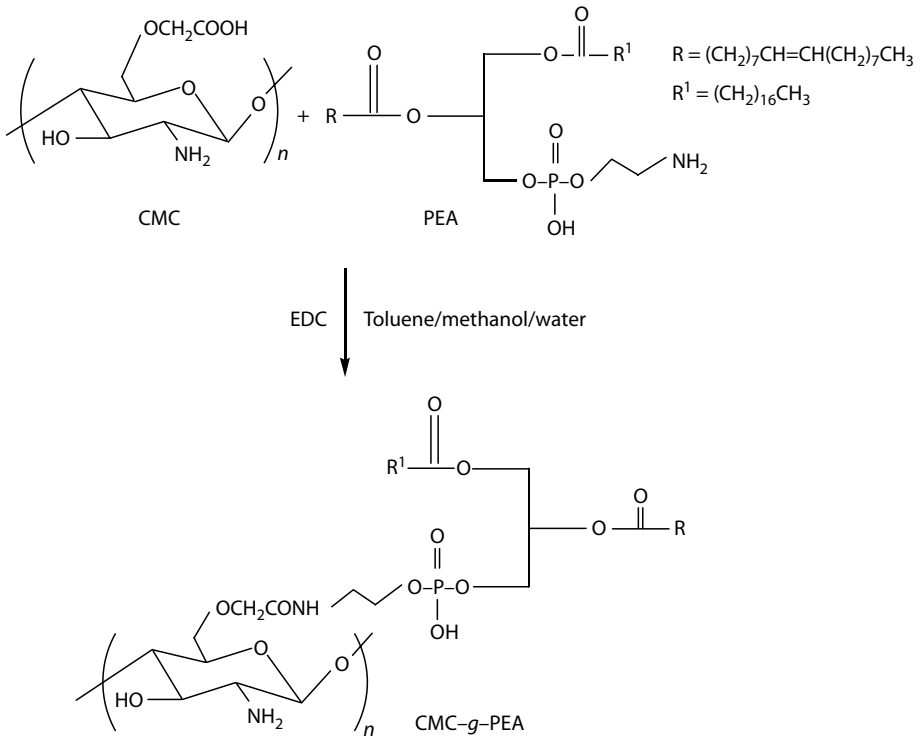


FIGURE 13.3 Reaction scheme for the preparation of CMC-*g*-PEA. (From Prabaharan, M. et al., *React. Funct. Polym.*, 67, 43, 2007. With permission.)

N-alkylation as shown in Figure 13.4. Their inclusion ability was examined by the use of *p*-nitrophenol and 3-methyl-4-nitrophenol as model compounds. The potent inclusion ability was observed on α-CD-grafted chitosan beads toward *p*-nitrophenol while 3-methyl-4-nitrophenol was not adsorbed on the beads. Controlled release study suggested that *p*-nitrophenol entrapped with α-CD-grafted chitosan beads was released slowly into the buffer and that equilibrium was reached after 15 h. In contrast to these results, chitosan beads, which have little ability to form inclusion complexes, released almost all of the *p*-nitrophenol within several hours.

Chen and Wang (2001) synthesized β-CD-*g*-chitosan by reacting β-CD with *p*-toluenesulfonyl chloride and then grafting with chitosan. The polymer inclusion complex of β-CD-*g*-chitosan with iodine was prepared and applied for slow release of radioactive iodine in rats. The experimental results showed that a nice bit of iodine was included with β-CD-*g*-chitosan and formed a stable inclusion complex while chitosan only had a little adsorbing ability of iodine. The stronger inclusion ability of β-CD-*g*-chitosan with iodine was caused by the special hydrophobic cavity structure of β-CD-*g*-chitosan. After the subcutaneous implantation of the polymer inclusion complex of β-CD-*g*-chitosan with iodine in rates, iodine exhibited the property of slow release. Prabaharan and Mano (2005a) synthesized a chitosan derivative bearing β-CD cavities to be used as a matrix for controlled drug release. This system provided a slower release of the entrapped hydrophobic drugs with a pH-responsive capability. Recently, thiolated carboxymethyl chitosan-*g*-β-CD (CMC-*g*-β-CD) was prepared as a mucoadhesive drug delivery carrier as shown in Figure 13.5 (Prabaharan and Gong 2008). The swelling study showed that the water uptake of thiolated CMC-*g*-β-CD was higher than that of the unmodified chitosan control. The adhesive properties of thiolated CMC-*g*-β-CD were evaluated in vitro on a freshly excised mouse mucosa, and a fivefold increase in the adhesion time was found in thiolated CMC-*g*-β-CD when compared with the unmodified chitosan control. Thiolated CMC-*g*-β-CD tablets provided a slower release of the entrapped hydrophobic model drug,

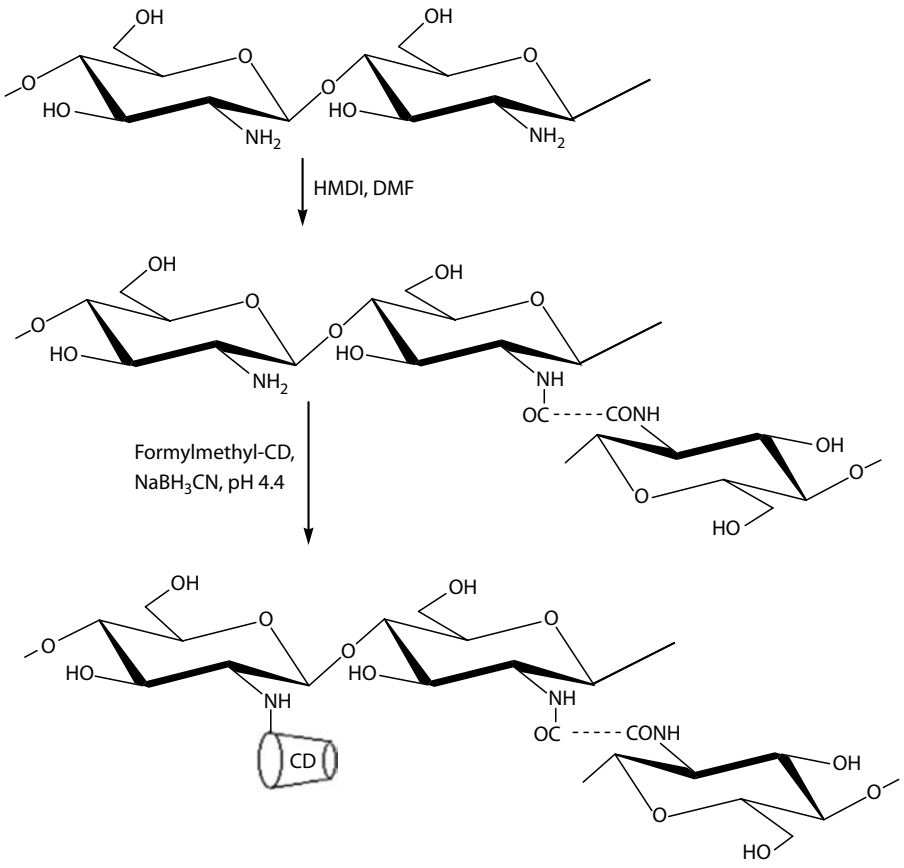


FIGURE 13.4 Reaction scheme for the preparation of α-CD-*g*-chitosan beads. (From Prabaharan, M. and Mano, J.F., *Carbohydr. Polym.*, 63, 153, 2006. With permission.)

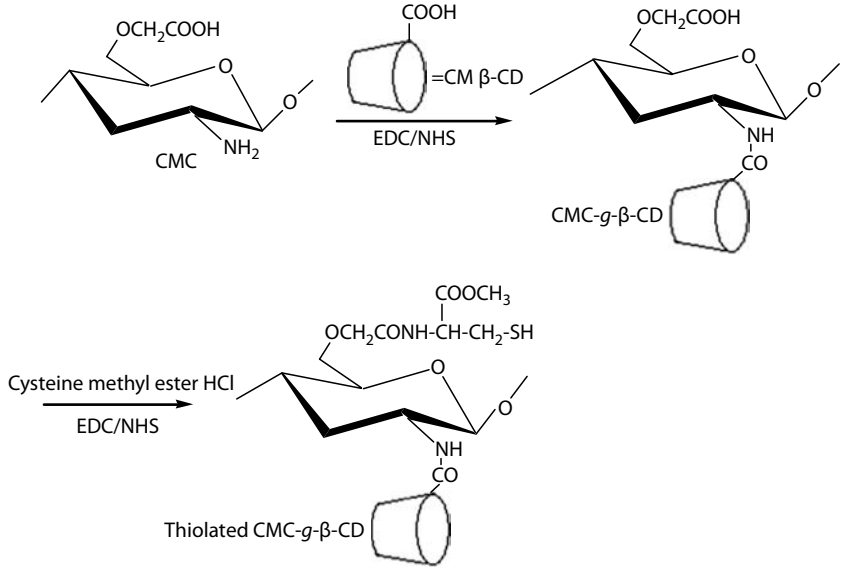


FIGURE 13.5 Synthesis of thiolated CMC-*g*-β-CD. (From Prabaharan, M. and Gong, S., *Carbohydr. Polym.*, 73, 117, 2008.)

ketoprofen, than the chitosan control, and the release behavior was influenced by the amounts of thiol groups present on the polymer chains.

13.3 IN GENE DELIVERY

13.3.1 Chitosan–Biomolecule Conjugates

An emerging application of chitosan-based colloidal systems is in the field of gene delivery. In an effort to avoid the problems commonly associated with viral-mediated transfection, a number of groups have been investigated cationic polymers as condensing agents for DNA. While such systems have shown some capabilities of facilitating transgene expression, polymer toxicity remains a major hurdle. Given the numerous studies reporting the low toxicity and biocompatibility of chitosan (Hirano et al. 1990), recent interest has been focused on this natural polycation as a gene delivery system. Lee et al. (1998) synthesized hydrophobically modified chitosan containing deoxycholic acid. The feasibility of chitosan self-aggregates as a delivery carrier for the transfection of genetic material in mammalian cells was also investigated. Self-aggregates can form charge complexes when mixed with plasmid DNA. These self-aggregate/DNA complexes are considered to be useful for transfer of genes into mammalian cells in vitro and served as a good delivery system composed of biodegradable polymeric materials. Recently, Cheong et al. (2009) prepared superparamagnetic iron oxide nanoparticles (SPIO)-loaded with water-soluble chitosan-linoleic acid nanoparticles (SCLNs) that formed gene complexes capable of localizing specifically to hepatocytes. This study confirmed that SCLNs labeled with ^{99m}Tc delivered into mice via intravenous injection accumulated mainly in the liver using nuclear and magnetic resonance imaging. In addition, the injection of the gene complexes into mice resulted in significantly increased expression of green fluorescence protein (pEGFP) in hepatocytes in vivo. Furthermore, gene silencing was effectively achieved by administration of gene complexes loaded with specific siRNAs.

13.3.2 Trimethyl Chitosan Oligomers

Florea et al. (2000) have prepared trimethyl chitosan oligomers (TMO) of 40% and 50% degrees of quaternization, respectively. These oligomers were examined for their potency as DNA carrier systems in two cell lines, COS-1 and Caco-2. The result showed that TMO proved to be superior to oligomeric chitosan in transfecting COS-1 cells, however, none of the used chitosan/DNA and lipofectin/DNA complexes was able to increase transfection efficiency in differentiated Caco-2 cells. Similarly, trimethylated chitosan (80% degree of quaternization) polymers, bearing antennary galactose residues through a 6-*O*-linked carboxymethyl group, were also examined as DNA carriers (Murata et al. 1997). The complexes were tested for Hep-G2 cells, which express the galactose receptor, and expression of β-galactosidase activity. The complexes efficiently transfected the Hep-G2 cells and the transfection efficiency was significantly inhibited in the presence of an inhibitor, indicating that the conjugates were specifically internalized via the galactose receptor present on the cellular surface of Hep-G2 cells.

13.3.3 Galactosylated Chitosan Derivatives

Park et al. (2000) prepared galactosylated chitosan-*g*-dextran DNA complexes. Galactose groups were chemically bound to chitosan for liver specificity and dextran was grafted to increase the complex stability in water. It was shown that this system could efficiently transfect Chang liver cells expressing the asialoglycoprotein (ASGR) receptor in vitro, indicating a specific interaction of the galactose ligands bound to chitosan with receptor. Leong et al. (1998) showed that chitosan–DNA nanospheres could transfect HEK 293, IB3, and HTE cell lines, through at lower levels than

lipofectamine controls. From this basic construct, several modifications were investigated. Though the coencapsulation of chloroquine significantly increased the transfection efficiency of another natural cationic polymer, gelatine, chitosan did not substantially benefit from the same coincorporation. Recently, Song et al. (2009) described the synthesis of novel galactosylated chitosan through etherization of chitosan and galactose using $BF_3 \cdot OEt_2$ as a promoter. Galactosylated chitosan showed great ability to form a complex with DNA and proper physicochemical properties for a gene carrier. Compared with chitosan/VRMFat-1, galactosylated chitosan/VRMFat-1 complexes showed a higher positive zeta potential.

13.4 IN TISSUE ENGINEERING

Tissue engineering aims to develop functional substitutes for damaged or diseased tissues through complex constructs of living cells, bioactive molecules, and three-dimensional porous scaffolds, which support cell attachment, proliferation, and differentiation. Together with the choice of appropriate cells and bioactive agents, the suitable material for the scaffold preparation plays a crucial role in the success of the application (Langer and Vacanti 1993). In this context, considerable attention has been given to chitosan because of its low cost, large-scale availability, antimicrobial activity, low toxicity, and biodegradability (Prabaharan and Mano 2005b). In tissue engineering applications, the cationic nature of chitosan is primarily responsible for electrostatic interactions with anionic glycosaminoglycans, proteoglycans, and other negatively charged molecules. This property is of great interest because a large number of cytokines/growth factors are linked to glycosaminoglycans, and a scaffold incorporating a chitosan–glycosaminoglycans complex may retain and concentrate growth factors secreted by colonizing cells. One of chitosan's most promising features is its excellent ability to be processed into porous structures for use in cell transplantation and tissue regeneration. However, the practical use of chitosan has been mainly restricted to the unmodified forms in tissue engineering applications. In recent years, numerous studies have been reported on the chemically modification of chitosan for tissue engineering applications.

Modification of chitosan for tissue engineering applications has been performed to introduce the specific recognition of cells by sugars. Sugar bound chitosans, such as chitosan with D- and L-fucose, and their specific interactions with lectin and cells have been reported (Li et al. 2000). Also, galactosylated chitosan prepared from lactobionic acid and chitosan as activating agents showed possibility of a synthetic extracellular matrix for hepatocyte attachment (Park et al. 2003). Generally, poly(α-hydroxyacid)s, homopolymers and copolymers based on glycolide and lactide have been widely used as a biomaterial in sutures, drug release systems, and tissue engineering due to their biocompatibility and biodegradability (Morita and Ikada 2002). Zhu et al. (2002) used a photosensitive heterobifunctional crosslinking agent attached to chitosan for coating onto poly(L-lactide) (PLA) film surfaces (Figure 13.6). Improved cell attachment was obtained with this approach whereas chitosan modified with heparin inhibited platelet adhesion and activation. The effect of cell adhesive peptides photochemically grafted onto chitosan surfaces on growing human endothelial cells was reported (Chung et al. 2002). Chitosan surfaces containing the grafted peptides were found to support the proliferation of human endothelial cells compared to chitosan itself without adherence. In another approach, the amino group of chitosan was reacted with the carboxylic acid group of amino acids (lysine, arginine, aspartic acid, phenylalanine) (Chung et al. 2002). These amino acid functionalized chitosan moieties were entrapped onto PLA surfaces.

Recently, Chenite et al. (2000) developed the thermally gelling chitosan system through neutralizing highly deacetylated chitosan solutions with glycerol phosphate to retain chitosan in solution at physiological pH. The thermogelling chitosan/glycerol phosphate solutions that can form a gel in body temperature are especially attractive as injectable implant systems in tissue engineering. Injectable implant systems offer the following advantages over the use of preformed scaffolds: liquid gels are able to fill any space or shape of a defect site, living cells and therapeutic agents are

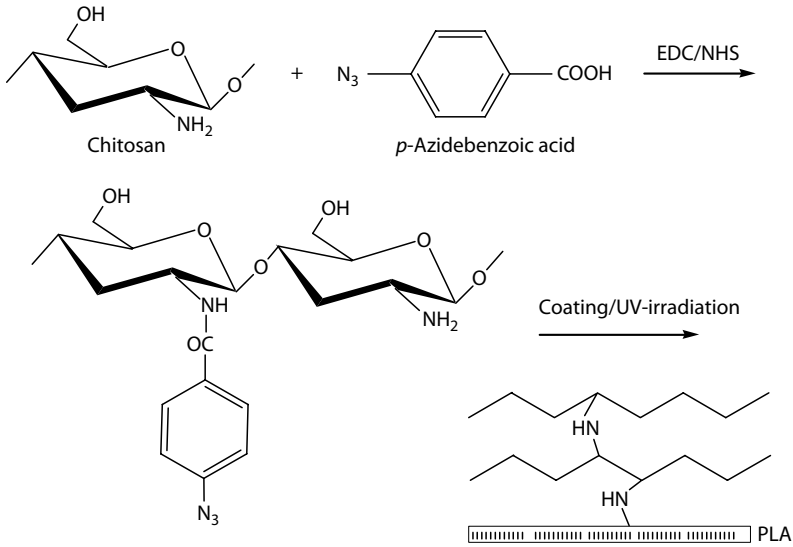
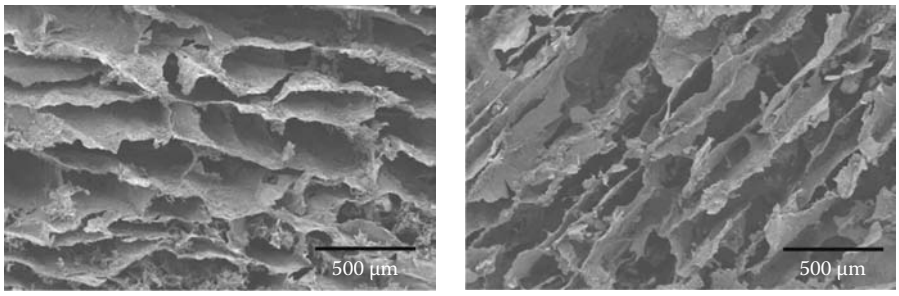


FIGURE 13.6 Immobilization of chitosan on PLA film surface. (From Zhu, A. et al., *Biomaterials*, 23, 4657, 2002. With permission.)

incorporated prior to the injection within the solution, and more importantly, the systems can be implanted in the site without surgery.

Three-dimensional biodegradable chitosan–nanohydroxyapatite composite scaffolds were reported for bone tissue engineering application (Thein-Han and Misra 2009). The nanocomposite scaffolds exhibited greater compression modulus, slower degradation rate, and reduced water uptake, but the water retention ability was similar to that of pure chitosan scaffolds. Recently, a microsphere-scaffold release system has been developed (Niu et al. 2009). Chitosan microspheres encapsulated with BMP-2-derived synthetic peptide were incorporated into nanohydroxyapatite and PLA-based matrix. In vitro degradation tests indicated that the addition of chitosan microspheres could increase the degradation rate of the scaffolds. Prabaharan and Jayakumar (2009) synthesized biodegradable scaffolds composed of chitosan-*g*-β-CD as synthetic extracellular matrices to fill the gap during the healing process (Figure 13.7). The morphology, swelling, and drug release properties of the scaffolds were found to be dependent on the extent of crosslinking density in the scaffolds. The drug dissolution profile showed that chitosan-*g*-β-CD scaffolds provided a slower release of the entrapped ketoprofen than chitosan scaffold due to the presence of β-CD.



(A) (B)

FIGURE 13.7 Morphology of the chitosan-*g*-β-CD scaffolds crosslinked with (A) 0.01 M glutaraldehyde and (B) 0.05 M glutaraldehyde. (From Prabaharan, M. and Jayakumar, R., *Int. J. Bio. Macromol.*, 44, 320, 2009.)

13.5 IN WOUND HEALING

13.5.1 EDTA-Grafted Chitosan

Chemically modified chitosan presents interesting properties for wound-healing applications, because chitosan derivatives can exhibit enhanced bacteriostatic activity with respect to pure chitosan. Ethylene diamine tetraacetic acid (EDTA) grafted onto chitosan increases the antibacterial activity of chitosan by complexing magnesium that under normal circumstances stabilizes the outer membrane of gram-negative bacteria (Valanta et al. 1998). The increase in chitosan antimicrobial activity is also observed with carboxymethyl chitosan, which makes essential transition metal ions unavailable for bacteria or binds to the negatively charged bacterial surface to disturb the cell membrane (Liu et al. 2001). Therefore, the grafted chitosans are used in wound healing systems, such as carboxymethyl chitosan for the reduction of periodontal pockets in dentistry and chitosan grafted with EDTA as a constituent of hydro- and hydro alcoholic gels for topical use.

13.5.2 Chitosan-Grafted Membranes

Hu et al. (2002) reported the grafting of acrylic acid onto ozone-treated poly (3-hydroxybutyric acid) (PHB) and poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) (PHBV) membranes. The resulting membranes were further grafted with chitosan or chitooligosaccharide via esterification. These chitosan- and chitooligosaccharides-grafted membranes showed antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, methicilin-resistant *Staphylococcus aureus*, and *S. aureus*. The results showed that the antibacterial activity to *S. aureus* was the lowest among these four bacteria. Acrylic acid grafting increased the biodegradability with *Alcaligens faecalis*, whereas chitosan and chitooligosaccharide grafting reduced the biodegradability. In addition, chitosan-*g*-PHBV membrane showed higher antibacterial activity and lower biodegradability than the chitooligosaccharides-grafted membrane.

Two anionic soluble monomers, mono(2-methacryloyl oxyethyl)acid phosphate and vinyl sulfonic acid sodium salt, were grafted onto chitosan to obtain copolymers with zwitterionic property (Jung et al. 1999). It was observed that the grafting reaction improved the antimicrobial activities of chitosan. Also, it was observed that the antimicrobial activity of chitosan and graft copolymers against *Candida albicans*, *Trichophyton rubrum*, and *Trichophyton violaceum* depends largely on the amount and type of grafted chains, as well as on the changes of pH. Yang et al. (2003) prepared polypropylene-*g*-acrylic acid using γ-ray irradiation, and studied its antimicrobial activity. In this study, the chitosan was immobilized onto this material using EDC. The antibacterial activity of the polypropylene was enhanced by the modification of γ-ray-radiation-induced grafting of acrylic acid and the immobilization of chitosan onto the polypropylene-*g*-acrylic acid modified polymer.

13.5.3 Chitosan/Poloxamer Semi-IPNs

Kim et al. (2007) evaluated the chitosan/poloxamer semi-interpenetrating polymer networks (SIPNs) for effective application as a wound dressing (Figure 13.8). The evaluations of their water uptake, water vapor permeation, and evaporative water loss showed optimal conditions for maintaining a properly moist environment conducive for wound dressing. Furthermore, the evaluation of chitosan/poloxamer SIPNs in enzymatic biodegradation provided necessary information for in vivo wound-dressing application. In vivo test, granulation tissue formation and wound contraction for the chitosan/poloxamer SIPNs and dehydroepiandrosterone-loaded chitosan/poloxamer SIPNs wound dressing were found to be faster than any other groups.

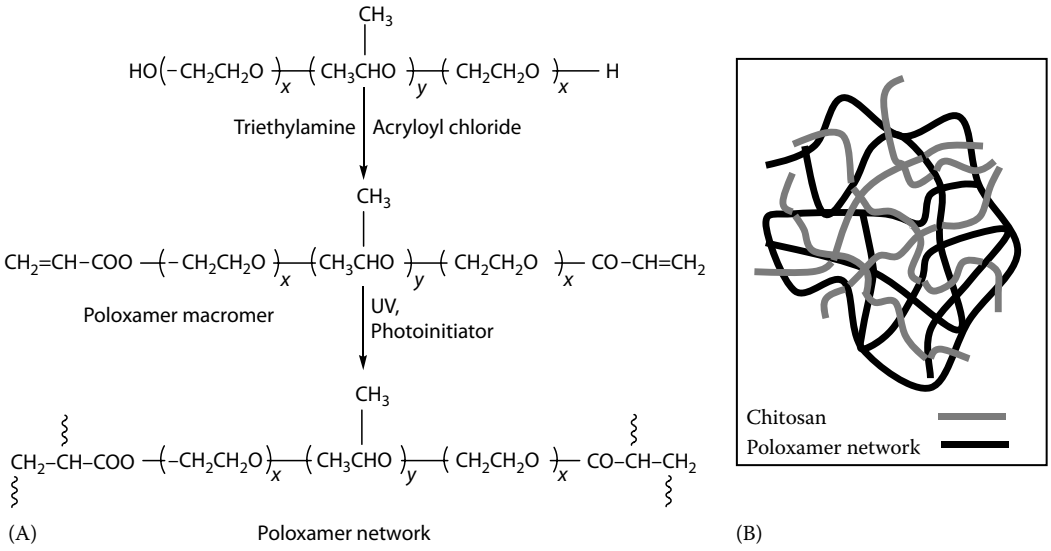


FIGURE 13.8 Synthesis scheme of poloxamer networks from poloxamer macromer (A) and structure of chitosan/poloxamer SIPNs (B). (From Kim, I.Y. et al., *Int. J. Pharm.*, 341, 35, 2007. With permission.)

13.5.4 Chitosan-Crosslinked Collagen Sponge

Recently, Wang et al. (2008) assessed the biochemical and biophysical improvement of the chitosan-crosslinked collagen sponge containing recombinant human aFGF (rhaFGF) as the new wound dressing for the therapeutic effect on the healing-impaired skin wound in streptozotocin-induced diabetic rats. The collagen crosslinked with chitosan provided several advantages required for wound dressing. Pre-clinical studies using type 1 diabetic rats with trauma skin wound indeed showed the most efficiently therapeutic effect of the new wound dressing containing FGF.

13.6 CONCLUSIONS

Over the last few years, an impressive number of chitosan derivatives have been developed for biomedical applications. The approach of chemical modification of chitosan with other functional materials such as lipids, cylodextrins, and thiolated and hydrophobic compounds has received potential importance in drug delivery since the resulted materials exhibit an improved drug loading and controlled release properties. Because of their cationic nature and chelating ability with DNA, chemically modified chitosan can be used as gene delivery carriers. Chitosan derivatives can be promising candidates as a wound healing and supporting material for tissue engineering applications because of their antibacterial activity, porous structure, gel forming properties, and high affinity for in vivo macromolecules.

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14 Enzymatic Modifications of Chitin and Chitosan

Yong Zhao, Wan-Taek Ju, and Ro-Dong Park

CONTENTS

14.1 INTRODUCTION

The high molecular weight of chitin and chitosan, which results in their poor solubility at neutral pH values, limits its potential uses in the fields of food, health, and agriculture. However, most of these limitations can be overcome by chitin and chitosan oligomer or monomer. In humans, chitin monomers are precursors of the disaccharide units in glycosaminoglycans (such as hyaluronic acid, chondroitin sulfate, and keratin sulfate), which are necessary to repair and maintain healthy cartilage and joint function. Chitosan oligomers in food and nutrition fields have the ability to improve food quality and human health progression. For large-scale production of chitin/chitosan oligomers, acid hydrolysis is commonly used to cleave glycosidic linkages of chitin and chitosan. However, the resultant oligomers are generally not considered to serve as safe bioactive materials because of a possibility of contamination of toxic chemical compounds. As a result, the enzymatic hydrolysis of chitin and chitosan has been proposed as a preferred method for the production of bioactive chitin/chitosan oligomers during the past few decades. The hydrolytic enzymes include cellulase, protease, lipase, pepsin, chitinase, chitosanase, and lysozyme, although only chitinase, chitosanase, and lysozyme are believed to be specific. These chitinolytic and chitosanolytic enzymes produced by different kinds of organisms have different action mode and size specificity.

In addition to their hydrolytic activity, some chitinolytic or chitosanolytic enzymes possess a certain level of transglycosylation ability. The transglycosylation activity of these enzymes implicates a great potential for the synthesis of desired chitin oligomers or polymers. During that time, the findings in the catalytic and substrate-binding mechanisms of chitinolytic enzymes as well as their sequence homology have been reviewed in detail by Fukamizo (2000). In this chapter, we briefly introduce the types and sources of several chitin and chitosan-modifying enzymes including chitinases, chitosanases, and chitin deacetylases as well as their applications in the modification of chitin/chitosan.

14.2 ENZYMATIC DEPOLYMERIZATION OF CHITIN

The enzymatic depolymerization of chitin by chitinases has been investigated for a few decades. Chitinases, a class of glycosyl hydrolyases, have been found in a variety of organisms ranging from bacteria to animals. Chitinases belong to two major families of carbohydrate enzymes, family 18 and family 19, based on the amino acid sequences (CAZY: http://www.cazy.org). Both families of enzymes differ in their amino acid sequences, three-dimensional structures, and catalytic mechanisms (Fukamizo 2000). Prior to the family classification, plant chitinases are divided into five classes on the basis of their primary structures. Classes I, II, and IV chitinases are included in family 19, whereas classes III and V belong to family 18.

According to their cleavage sites, chitinases can be classified into two major categories, endo-chitinases and exo-chitinases. As shown in Figure 14.1, endo-chitinases (EC 3.2.1.14) generally cleave the linkage of GlcNAc-GlcNAc, GlcN-GlcNAc, and GlcNAc-GlcN in chitin to release smaller, soluble chitin oligomers of variable size. Exo-chitinases can be divided into two subcategories: *N*,*N*′-diacetylchitobiohydrolase (chitobiase, EC 3.2.1.29) and β-*N*-acetylglucosaminidase (GlcNAc-ase, EC 3.2.1.30). Chitobiases catalyze the progressive release of chitobiose either from the nonreducing or reducing end of chitin. GlcNAc-ases progressively break down chitin polymer or oligomers from the nonreducing or reducing end of the molecule, releasing β-D-GlcNAc or α-D-GlcNAc, respectively (Figure 14.1).

Endo-chitinases have been well studied till now. For instance, Jung and colleagues have described an endo-chitinase from *Paenibacillus illinoisensis* KJA-424. The enzyme hydrolyzed $(GlcNAc)_4$ to

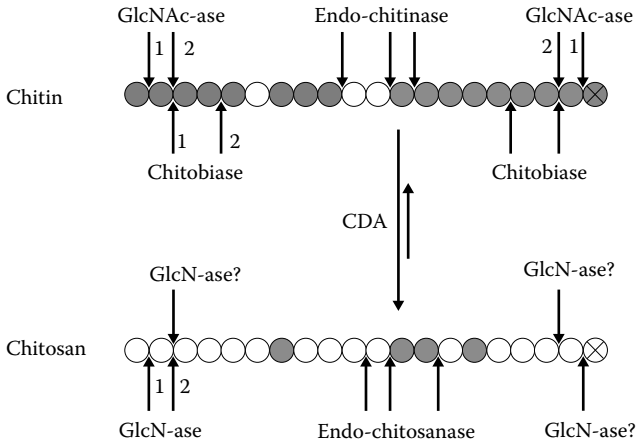

FIGURE 14.1 An overview of various chitin- and chitosan-modifying enzymes. GlcNAc and GlcN are represented by open and shaded circles, respectively. The reducing end residue was indicated by the circle containing X inside. Chitin is hydrolyzed by endo-chitinases, GlcNAc-ase and chitobiase. Endo-chitinases randomly cleave not only the linkage of GlcNAc-GlcNAc but also those of GlcN-GlcNAc and GlcNAc-GlcN of chitin chain to release its oligomers. GlcNAc-ase hydrolyze chitin from either non-reducing end or reducing end, releasing β-D-GlcNAc or α-D-GlcNAc, respectively. On the other hand, chitin is hydrolyzed by chitobiase from either non-reducing end or reducing end, releasing β-$(GlcNAc)_2$ or α-$(GlcNAc)_2$, respectively. The cleavage position was indicated by the arrows. The numbers 1 and 2 indicate the sequence by which chitin was hydrolyzed. Chitosan is hydrolyzed by endo-/exo-chitosanases. Endo-chitosanases randomly cleave the linkage of GlcN-GlcN, GlcN-GlcNAc and GlcNAc-GlcN in chitosan and release chitosan oligomers. So far, GlcN-ase, an exo-chitosanase, can only hydrolyze the linkage of non-reducing end GlcN residues. The GlcN-ase together with a question mark, indicates the unknown. CDA is an enzyme that can either deacetylate chitin to chitosan or catalyze the reverse reaction from chitosan to chitin. (Modified from El Gueddari, N.E. et al., Substrates and products of chitin- and chitosan-modifying enzymes. In S. Şenel, K. M. Vårum, M. M. Şumnu, and A. A. Hincal (Eds.), *Advances in Chitin Science*, Vol. 10, Hacettepe University Press, Ankara, Turkey, 2007, 119–126.)

$(GlcNAc)_2$ and $(GlcNAc)_5$ to $(GlcNAc)_2$ and $(GlcNAc)_3$, while this enzyme showed no activity toward $(GlcNAc)_2$ and $(GlcNAc)_3$, suggesting that at least four GlcNAc residues in chitin substrates are necessary for recognition by this enzyme (Jung et al. 2005).

Exo-chitinases have also been well studied. As an example, Kuk and colleagues have reported that two types of exo-chitinases (GlcNAc-ase and chitobiase) are associated with enzymatic preparation of *Aeromonas* sp. GJ-18. The activities of these enzymes are strongly influenced by the reaction temperature when swollen chitin was used as the substrate (Kuk et al. 2005).

Compared with nonreducing-end-acting exo-chitinases, a rather small number of reducing-end-acting ones have been described, although it already becomes apparent that they have unique binding and catalytic mode in their substrate (Aronson and Halloran 2006; Honda et al. 2008).

The bacterium, *Serratia marcescens*, is one of the most extensively studied multiple chitinases producer (Horn et al. 2006; Suzuki et al. 2002; Vaaje-Kolstad et al. 2005). This bacterium possesses three chitinases ChiA, ChiB, and ChiC, as well as one chitin-binding protein CBP21. ChiC often occurs in two forms in cultures of this bacterium: the complete protein, sometimes called ChiC1, and a proteolytically truncated variant, called ChiC2, which lacks the two putative chitin-binding domains. ChiC is suggested as an endo-chitinase and degrade chitin in a nonprogressive mode.

Different from ChiC, ChiA and ChiB were considered to digest chitin chains in the opposite direction. ChiA was proposed to degrade the chitin chain from the reducing end, producing mainly α-$(GlcNAc)_2$, whereas ChiB, from the nonreducing end, producing mainly β-$(GlcNAc)_2$ (Suzuki et al. 2002). Although inactive on chitin substrate, the chitin-binding protein CBP21 plays a key role in enzymatic degradation of insoluble crystalline substrate through interfering with chitin crystal packing, thus increasing substrate accessibility and enzyme efficiency and hence chitin degradation (Vaaje-Kolstad et al. 2005).

Specific combinations of chitinolytic enzymes would be necessary to obtain the desired chain length and anomer-types of chitin oligomers or monomers. For instance, production of $(GlcNAc)_n$ ($n > 2$) requires high levels of endo-chitinase and low levels of exo-chitinase, whereas the production of GlcNAc or $(GlcNAc)_2$ requires higher proportion of GlcNAc-ase or chitobiase, respectively.

Besides chitinases, several other commercial enzymes possess chitinolytic activity. For instance, lysozymes, chitosanases, cellulase, and *N*-acetylhexosaminidase also have the ability to hydrolyze chitin polymers, even though their substrate specificities are different from that of chitinases. Furthermore, chitinases from different sources may exhibit different chitin modification modes, facilitating its potential use in industrial applications.

14.3 ENZYMATIC DEPOLYMERIZATION OF CHITOSAN

According to their cleavage sites, chitosanases can be classified into two major categories. As shown in Figure 14.1, endo-chitosanases (EC 3.2.1.132) cleave chitosan at random and produce chitooligosaccharides. Exo-chitosanases are usually called exo-β-D-glucosaminidases (GlcN-ase), which cleave GlcN residues continuously from the nonreducing end of the substrate (Figure 14.1). So far, most of the reported chitosanases are endo-type chitosanases. Based on the amino acid sequence, chitosanases belong to family 5, 7, 8, 46, 75, and 80 glycoside hydrolases (CAZY: http://www.cazy.org). On the other hand, chitosanases can be classified into four subclasses according to their substrate specificity: subclass I split GlcNAc-GlcN and GlcN-GlcN bonds, subclass II split only the GlcN-GlcN bonds, subclass III split GlcN-GlcN and GlcN-GlcNAc bonds, and subclass IV chitosanases split GlcNAc-GlcN, GlcN-GlcNAc as well as GlcN-GlcN bonds (Mitsutomi et al. 2008). In every subclass, chitosanases commonly hydrolyze the β-1,4-linkage when -1 or $+1$ subsites are at least occupied by GlcN residues (Jung et al. 2006).

In bacteria, *Bacillus* is the main genus for secreting chitosanases (Fukamizo et al. 2005; Gao et al. 2008; Jo et al. 2003). The chitosanases from the *Bacillus* sp. required substrates with three or more GlcN or GlcNAc residues for the expression of activity. Gao and colleagues purified an endo-chitosanase from *Bacillus cereus* D-11 and studied its cleavage pattern using chitooligosaccharide

alcohols (Gao et al. 2009). The chitosanase requires at least five GlcN residues for enzymatic hydrolysis and cleaves $(GlcN)_4GlcNOH$ into $(GlcN)_3$ + GlcNGlcNOH; $(GlcN)_5GlcNOH$ into $(GlcN)_3$ + $(GlcN)_2GlcNOH$ and $(GlcN)_4$ + GlcNGlcNOH, respectively. From the cleavage patterns of the chitooligosaccharide alcohols, it was suggested that *B. cereus* D-11 chitosanase should have five subsites, –3, –2, –1, +1, and +2.

In contrast to endo-chitosanases, there are only a few reports on exo-chitosanases. Nanjo and colleagues were the first to purify and characterize an exo-chitosanase from *Nocardia orientalis* (Nanjo et al. 1990). Afterwards, exo-chitosanases were characterized from a few organisms, however, all of them seem to exhibit a similar catalytic mode, that is, these enzymes could only cleave the linkage of GlcN-GlcN or GlcN-GlcNAc from the nonreducing end of chitosan and chitosan oligomers, releasing GlcN as the only product (Cote et al. 2006; Jung et al. 2006; Nanjo et al. 1990; Nogawa et al. 1998; Zhang et al. 2000). There is no report to date about the exo-chitosanases that could release $(GlcN)_2$ from nonreducing end or release GlcN or $(GlcN)_2$ from the reducing end (Figure 14.1). Recently, Yao and colleagues have successfully described a new approach to convert the endo-chitosanase from *B. circulans* MH-K1 to an exo-chitosanase through structural simulation of exo-type glycoside hydrolases. They designed and inserted peptides (PCLGG) and (SRTCKP) after the positions of Asp115 and Thr222, respectively. The results showed that only $(GlcN)_2$ was released from chitosan by the mutated enzyme (Yao et al. 2008).

14.4 ENZYMATIC TRANSGLYCOSYLATION APPROACHES

In addition to their hydrolytic activity, chitinases, chitosanases, and lysozymes (typically, exo-type enzymes) possess certain levels of transglycosylation ability. For instance, Usui and colleagues observed the accumulation of $(GlcNAc)_6$ and $(GlcNAc)_2$ from $(GlcNAc)_4$ and the accumulation of $(GlcNAc)_7$ and $(GlcNAc)_3$ from $(GlcNAc)_5$ in the presence of *Nocardia orientalis* chitinase (Usui et al. 1987). In another report, an exo-chitosanase from *Aspergillus fumigatus* S-26, which is essentially a hydrolase, also catalyzes a transglycosylation reaction, resulting in the one-residue elongated chitosan oligomers (Jung et al. 2006). More recently, Li and colleagues described a novel application of *Bacillus* sp. chitinase for the chemoenzymatic synthesis of *N*-linked neoglycoproteins. This enzyme was able to glycosylate asparagine-linked GlcNAc in a regio- and stereo-specific manner (Li et al. 2008).

Under conditions that favor a reversal of their hydrolytic activity, chitinases and chitosanases can be used successfully to promote the synthesis of chitooligosaccharides. This may be achieved either by shifting the equilibrium using high substrate concentrations or by using activated glycosyl donors. For instance, Usui and colleagues observed an effective chain elongation from $(GlcNAc)_2$ to $(GlcNAc)_{6-7}$ using egg lysozyme in the presence of 30% ammonium sulfate (Usui et al. 1990). Hsiao and colleagues enhanced the transglycosylation reaction by using a reversed micellar microreactors, which could efficiently control the water activity in the enzymatic reaction (Hsiao et al. 2008).

A more sophisticated method has been tried using glycosynthases, specifically mutated glycosidases that efficiently synthesize chitooligosacchadrides but do not hydrolyze them, and represents a promising solution to these problems (Perugino et al. 2004). The mutated enzymes, in which at the active site the catalytic nucleophile (Asp or Glu) is replaced by a non-nucleophilic residue (typically Ala, Ser or Gly), lose their hydrolytic activity but retain transglycosylation activity with suitably activated donors such as glycosyl fluorides, with inverted anomeric configuration in a virtually quantitative yield. Very recently, this approach has been extended to inverting glycosidases and hexosaminidases (Honda et al. 2008).

14.5 ENZYMATIC DEACETYLATION OF CHITIN AND CHITOSAN

Industrial production of chitosan from chitin is usually carried out by high concentration of NaOH at high temperatures. Alternatively, the conversion from chitin to chitosan can be achieved by chitin deacetylase (CDA, E.C.3.5.1.41). CDA is one of the members of the carbohydrate esterase

family 4 (CE-4s) as defined in the CAZY database (http://afmb.cnrs-mrs.fr/~cazy/CAZY). Members of this family share a conserved region in their primary structure, which has been assigned as the "NodB homology domain" or "polysaccharide deacetylase domain." Besides chitin deacetylase, there are several other members in this family, including NodB protein (EC 3.5.1.-) (John et al. 1993) and peptidoglycan deacetylase (EC 3.1.1.-) (Vollmer and Tomasz 2000).

The main issue existed for bioconversion of chitin to chitosan is that CDA cannot effectively deacetylate natural crystalline chitin (Zhao et al. 2010). Win and Stevens reported that when treated with CDA, natural chitin powders exhibited only about 1% increase in their overall degree of deacetylation even on prolonged incubation (Win and Stevens 2001). It was postulated that the 1% enzymatic deacetylation may come from the peripherally located *N*-acetyl groups. Nevertheless, it is the only 1% enzymatically deacetylated chitin particles, namely "chit-in-osan," that exhibited a strongly increased binding capacity toward ovalbumin, while maintaining the rigidity and insolubility of chitin in a moderate acidic environment (Aye et al. 2006).

Although not effective on natural chitin, CDA is indeed a good biocatalyst for the modification of chitin oligomers. The pathways of enzymatic deacetylation of chitin oligomers have been overviewed as shown in Figure 14.2. CDAs from different sources show different enzymatic action patterns on chitin substrate. Only two types of CDAs have been studied: CDAs from Zygomycetes and those from Deuteromycetes. The action mode of CDA from *Mucor rouxii*, a Zygomycetes, has been studied on substrate of partially *N*-acetylated chitosans (Martinou et al. 1998) and *N*-acetylchitooligosaccharides (DP 1-7) (Tsigos et al. 1999). It was found that the exo-type enzyme hydrolyzes the acetyl groups of the substrate of either chitosan polymers or chitin oligomers according to a multiple attack mechanism in which a number of sequential deacetylations occurs after once binding of the enzyme to a chitin chain, as shown in Figure 14.2C. The enzyme could effectively deacetylate chitin oligomers with a DP higher than 2 and the first deacetylation takes place at the nonreducing-end residue of the oligomers. Among chitin oligomers (DP 2-7) tested, $(GlcNAc)_4$ and $(GlcNAc)_5$ could be fully deacetylated, whereas the reducing-end residue of $(GlcNAc)_3$, $(GlcNAc)_6$ and $(GlcNAc)_7$ always remains intact (Tsigos et al. 1999).

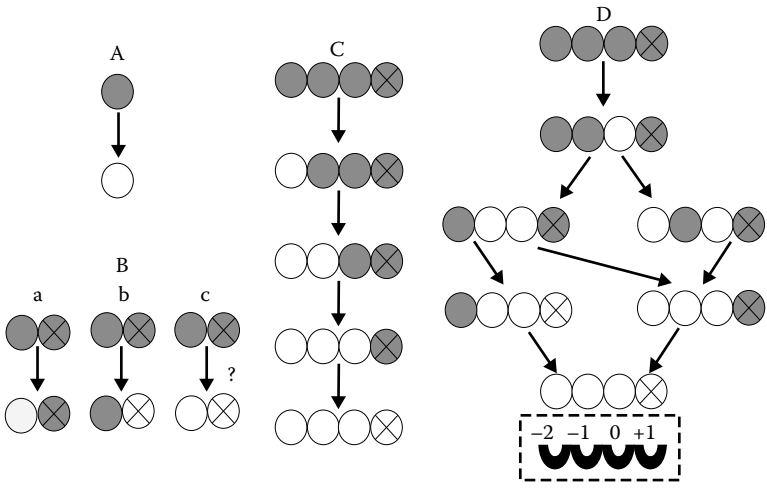

FIGURE 14.2 Enzymatic deacetylation of chitin monomer and chitin oligomers. A, GlcNAc is deacetylated to GlcN (Tanaka et al., 2004); B, GlcNAc-GlcNAc can be deacetylated to either (a) GlcN-GlcNAc (Tanaka et al. 2004) or (b) GlcNAc-GlcN (Kadokura et al. 2007) whereas (c) deacetylation from GlcNAc-GlcNAc to GlcN-GlcN is unknown; C, Chitin tetramer is deacetylated by *M. rouxii* CDA from the nonreducing end in a progressive multiple attack mode (Tsigos et al. 1999); D, Chitin tetramer is deacetylated by CDA from *C. lindemuthianum* in a multiple chain mode and four subsites are suggested as −2, −1, 0, +1. Among them, subsite 0 is responsible for the catalysis (Hekmat et al., 2003; Tokuyasu et al. 2000a).

Besides *M. rouxii* CDA, another well-studied CDA is that from *Colletotrichum lindemuthianum*, a Deuteromycetes (ATCC 56676). The purified enzyme could fully deacetylate $(GlcNAc)_3$ and $(GlcNAc)_4$, while it could only deacetylate the nonreducing-end GlcNAc residue of chitobiose (Tokuyasu et al. 1997). In a further study, Tokuyasu and colleagues found that $(GlcNAc)_4$ could be exclusively deacetylated to the product of GlcNAcGlcNAcGlcNGlcNAc by CDAH (the recombinant nonglycosylated CDA from *C. lindemuthianum*) in an initial deacetylation process. For a better understanding the reaction mechanism, it was proposed that the enzyme have four subsites (−2, −1, 0 and +1), as shown in Figure 14.2D. The enzyme strongly recognizes a sequence of four GlcNAc residues of the substrate, and the *N*-acetyl group in the GlcNAc residue positioned at subsite 0 is exclusively deacetylated (Tokuyasu et al. 2000a). Recently, the presence of four subsites CDAH that interact with GlcNAc residues was experimentally confirmed. The turnover number is independent of *n* and represents the intrinsic rate constant for the hydrolysis of the acetamido group in subsite 0. The steady-state kinetic parameter for the second deacetylation reaction of $(GlcNAc)_4$ were also determined using (GlcNAcGlcNAcGlcNGlcNAc) as the substrate. The results suggest that the mono-deacetylated substrate binds strongly in a nonproductive mode occupying all four subsites, thereby delaying the second deacetylation reaction (Hekmat et al. 2003).

CDA also exhibits a significant biological role for the plant pathogenic fungi during infection. For instance, *C. lindemuthianum* CDA could partially deacetylate chitin exposed on the surface of penetrating hyphae in a specific manner that may prevent recognition by either chitinases or chitosanases of plants (El Gueddari et al. 2002).

Besides CDAs, a few chitooligosaccharide deacetylases (EC 3.5.1.-) have been reported with a diverse substrate specificities. For instance, Tanaka and colleagues reported that one enzyme from *Thermococcus kodakaraensis* KOD1 was capable of conversion of GlcNAc to GlcN (Figure 14.2A). This bacterium also secreted another enzyme that was responsible for the deacetylation of $(GlcNAc)_2$ to GlcNGlcNAc (Figure 14.2Ba) (Tanaka et al. 2004). In another report, Kadokura and colleagues isolated a chitin-degrading bacterial strain, KN1699, which produced an extracellular chitooligosaccharide deacetylase. This enzyme specifically deacetylated GlcNAcGlcNAc to GlcNAcGlcN (Figure 14.2Bb) (Kadokura et al. 2007). However, there is no report on CDA that could fully deacetylate $(GlcNAc)_2$ to $(GlcN)_2$, as shown in Figure 14.2Bc.

14.6 ENZYMATIC ACETYLATION OF CHITIN AND CHITOSAN

In contrast to CDA's hydrolysis abilities, CDA's capability to catalyze chitosan and chitin is scarcely reported. Tokuyasu and colleagues have described that the CDA from *C. lindemuthianum* could acetylate free amino sugar residues into *N*-acetylated form, such as from $(GlcN)_2$ to GlcNAcGlcN (Tokuyasu et al. 1999) and from $(GlcN)_4$ to GlcNAcGlcNAcGlcNAcGlcN (Tokuyasu et al. 2000b).

14.7 CONCLUSIONS

Significant progress has been made in the modification of chitin and chitosan over the past few years for increasing the potential applications of these modified molecules in various fields. However, much more work is required to accomplish the enzymatic modification of chitin and chitosan. For example, the bioconversion of natural crystalline chitin to chitosan by chitin deacetylases is still a challenge for us, even though several approaches have been tried. In addition, our understanding of the transglycosylation by glycosidases is still not enough. To some extent, a poor understanding may be the result of the impressive number of glycosidases and chitin deacetylases available for studies. Thus, the discovery and characterization of novel chitin/chitosan-modifying enzymes is welcome in the near future.

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Part IV

Biological Activities of Chitin and Chitosan Derivatives

15 Antimicrobial Activity of Chitin, Chitosan, and Their Oligosaccharides

Joydeep Dutta and Pradip Kumar Dutta

CONTENTS

15.1 INTRODUCTION

The antimicrobial activity of any substance is always directed toward its applicability. Very recently, research is focused on the development of materials with film-forming capacity and antimicrobial properties that help improve food safety and shelf life. Antimicrobial packaging is one of the most promising active packaging systems that have been found to be highly effective in killing or inhibiting spoilage and pathogenic microorganisms that contaminate foods (Salleh et al. 2007). In this context, chitosan films have shown great promise for their application in food preservation. It is well known that microbial alternations are responsible for the enormous losses in food and hence, over the years, various chemical and physical processes have been developed to extend the shelf life of foods. The antimicrobial activity limits or prevents microbial growth by extending the lag period and reducing the growth rate or decreasing live counts of microorganisms (Han 2000). Currently, application of an antimicrobial packaging system for food is limited due to the availability of suitable antimicrobials, new polymer materials, regulatory concerns, and appropriate testing methods (Jin and Zhang 2008). In particular, polymeric bioactive films laced with an assortment of antimicrobial agents have been found to be very effective and practical in such applications. Till date, a number of articles have been published describing the nature of different materials used in making films and their effectiveness in food preservation (Alvarez 2000, Appendini and Hotchkiss 2002, Cha and Chinnan 2004, Coma 2008a,b, Cooksey 2005, Cutter 2002a,b, 2006, Dutta et al. 2009, Ozdemir and Floros 2004, Quintavalla and Vicini 2002, Suppakul et al. 2003). This chapter aims to summarize all the known methods of formation of chitosan-based films with antimicrobial properties and discuss their mode of action and applicability, particularly to extend the shelf life of products.

15.2 CHITIN, CHITOSAN, AND THEIR OLIGOSACCHARIDES VERSUS OTHER POLYMERS IN ANTIMICROBIAL ACTIVITY

The functional efficiency of a substance strongly depends on the nature of its components, film composition, and structure. The choice of film-forming substance and/or active additive is made based on the objective of its application, the nature of the food product, and/or the application method. So, polysaccharide films are made of starch, alginate, cellulose ethers, chitosan, carrageenan, or pectins that impart hardness, crispness, compactness, thickening quality, viscosity, adhesiveness, and gel-forming ability to a variety of films (Baldwin et al. 1995, Ben and Kurth 1995, Cutter and Sumner 2002, Glicksman 1983, Nisperos-Carriedo 1994, Whistler and Daniel 1990). While lipid films could produce anaerobic conditions, polysaccharide-derived films exhibit excellent gas permeability properties, resulting in desirable modified atmospheres that enhance the shelf life of the product without creating anaerobic conditions (Baldwin et al. 1995). Additionally, polysaccharide films and coatings can be used to extend the shelf life of muscle foods by preventing dehydration, oxidative rancidity, and surface browning (Nisperos-Carriedo 1994). Because of the make up of the polymer chains, polysaccharide films can exhibit low gas permeability; but their hydrophilic nature makes them poor barriers of water vapor (Ben and Kurth 1995). Interestingly, polysaccharide films have been available commercially for the Japanese meat industry for a number of years (Labell 1991). When applied to wrapped meat products and subjected to smoking and steam, the polysaccharide film actually dissolves and becomes

integrated into the meat surface. Meats treated with the polysaccharide film in this manner exhibited higher yields, improved structure and texture, and reduced moisture loss (Labell 1991, Stollman et al. 1994).

Particularly, the use of bio-based, polymer-based films as antimicrobial delivery systems to reduce undesirable bacteria in foodstuffs is not a novel concept. Various approaches have been proposed and demonstrated for the use of these films to deliver compounds to a variety of food surfaces, including muscle foods. These types of films, gels, or coatings are receiving considerable attention since they satisfy the consumer demand for products made using sustainable materials and/or recyclability (Durango et al. 2006). Numerous researchers have demonstrated that antimicrobial compounds such as organic acids (acetic, propionic, benzoic, sorbic, lactic, lauric), potassium sorbate, bacteriocins (nisin, lacticin), grape seed extracts, spice extracts (thymol, *p*-cymene, cinnamaldehyde), thiosulfinates (allicin), enzymes (peroxidase, lysozyme), proteins (conalbumin), isothiocyanates (allylisothiocyanate), antibiotics (imazalil), fungicides (benomyl), chelating agents (EDTA), metals (silver), or parabens (heptylparaben) could be added to edible films to reduce bacteria in solution, on culture media, or on a variety of muscle foods (Cha and Chinnan 2004, Cutter 2002a,b; Devlieghere et al. 2000, Han 2000). Additional studies have also demonstrated that antifungal compounds, organic acids, potassium sorbate, or the bacteriocin nisin were more effective for reducing levels of foodborne organisms when immobilized or incorporated into an edible film made from starch, carrageenan, or alginate, and applied to meat surfaces than when these antimicrobial compounds were applied via solution (Baron and Sumner 1994, Cutter and Siragusa 1996, 1997, Dawson et al. 1996, Meyer et al. 1959, Padgett et al. 1998, Siragusa and Dickson 1992, 1993). For example, Meyer et al. (1959) were among the first researchers to demonstrate that antibiotics and antifungal compounds could be added to a carrageenan film to reduce bacteria by 2 log 10 (99%) on poultry. Antimycotic agents also have been incorporated into edible coatings from waxes and cellulose ethers (Hotchkiss 1995). Siragusa and Dickson (1992, 1993) demonstrated that organic acids were more efficacious for reducing levels of *Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherichia coli* O157:H7 when immobilized in calcium alginate and applied to beef carcass tissue than when these materials were applied alone. Baron and Sumner (1994) demonstrated that potassium sorbate and lactic acid could be incorporated into an edible cornstarch film to inhibit *S. typhimurium* and *E. coli* O157:H7 on poultry. Cutter and Siragusa (1996, 1997) reported that immobilization of the bacteriocin nisin in calcium alginate gels not only resulted in greater reductions of bacterial populations on lean and adipose beef surfaces, but also resulted in greater and sustained bacteriocin activity when the tissues were ground and stored under refrigerated conditions for up to 7 days, as compared to nisin-only controls. Fang and Lin (1994) applied calcium alginate–containing nisin to pork and also demonstrated significant reductions in pathogen populations. In other similar studies, Dawson et al. (1996) and Padgett et al. (1998) demonstrated that nisin and lysozyme could be incorporated into edible heat-set and cast films made from corn zein or soy protein and exhibit activity against *E. coli* and *Lactobacillus plantarum*. Hoffman et al. (2000) demonstrated that corn zein films impregnated with EDTA, lauric acid, nisin, and combinations of the three compounds resulted in significant reductions of *L. monocytogenes* in solution. Franklin et al. (2004) also demonstrated that *L. monocytogenes* could be inhibited >2 log 10 (99%) on the surface of hot dogs using plastic-based packaging films treated with methylcellulose/hydroxypropyl methylcellulose-based solutions containing nisin. A number of additional studies have demonstrated that antimicrobial compounds can be incorporated into edible films made from animal-derived proteins (i.e., collagen, gelatin, and chitosan). Gill (2000) applied gelatin-based coatings containing lysozyme, nisin, and EDTA to ham and sausage to control spoilage and pathogenic organisms such as *Lactobacillus sake*, *Leuconostoc mesenteroides*, *L. monocytogenes*, and *S. typhimurium*. Cutter and Siragusa (1997) demonstrated that the addition of the bacteriocins, nisin, into a bovine-derived ibrinogen/thrombin-based gel, known as Fibrimex, may provide an added antimicrobial advantage to restructured raw meat products that incorporate surface tissues into product interior

or as a delivery system for antimicrobials to meat surfaces. Cutter and Miller (2004) also demonstrated that *Brochothrix thermosphacta* and *L. monocytogenes* were inhibited on hot dog surfaces subjected to temperature variations as well as long-term refrigerated storage following treatments with nisin-incorporated collagen (Coffi) films (NICF). Another study addressed the application of antimicrobials with milk proteins for edible films and coatings for foods. Whey protein films were treated with essential oils of oregano, rosemary, and garlic and evaluated against *E. coli* O157:H7, *Staphylococcus aureus*, *Salmonella enteritidis*, *L. monocytogenes*, and *L. plantarum* (Seydim 2006). While these studies demonstrated the efficacy of the antimicrobial compound in vitro, additional studies with surface-inoculated foodstuffs are warranted. A number of additional studies have addressed the production of edible films from agricultural sources and interaction with antimicrobials. For example, pullulan films can be produced by fungi during fermentation. In a recent study, lysozyme and disodium EDTA were incorporated into pullulan films made from the exopolysaccharides of *Aureobasidium pulluans* and evaluated for antimicrobial effectiveness against *E. coli* and *L. plantarum* (Kandemir et al. 2005). The resulting films were composed of glucans and polysaccharides, had a neutral pH, and were water soluble, transparent, and exhibited low oxygen permeability. In their studies, the researchers demonstrated that these antimicrobial films were stable for up to 21 days during cold storage and could inhibit *E. coli* under laboratory conditions. As evidenced by the information presented above, the application of biopolymers, bio-based polymers, edible gels, films, or coatings incorporated with food preservatives and/or natural antimicrobial compounds have the potential to find practical applications in the food industry. This specific information demonstrates the feasibility and applicability for incorporating various antimicrobial compounds with a range of inhibitory activity directly into bio-based or edible packaging materials for use in controlling food spoilage as well as enhancing microbial safety of muscle foods. Eventually, chitin, chitosan, and their oligosaccharides are also used in these additives for antimicrobial activity.

15.3 PREPARATION OF CHITOSAN-BASED ANTIMICROBIAL FILMS/COATINGS

Various methods are employed to prepare antimicrobial chitosan films and coatings for food packaging applications. Solution casting method is one of the popular methods. As a general practice, chitosan films are prepared by using various kinds of cross-linkers. Some typical preparative techniques are enumerated below.

15.3.1 Preparation of Starch/Chitosan Blend Film under the Action of Irradiation (Zhai et al. 2004)

Chitosan solution was prepared in acetic acid solution (chitosan:acetic acid = 5:4). Starch powder was mixed with glycerol homogenously with the above prepared chitosan solution to form 15% starch and chitosan semisolid gel-like mixtures by heating at 100°C for 2 h. The gel-like mixtures in hot state were cold pressed to prepare wet starch/chitosan films.

In order to produce a kind of antibacterial films, the wet films prepared through above methods were irradiated further at room temperature by electron beam (EB). The wet starch/chitosan films were dried at room temperature to gain starch/chitosan films. In another method, preparation of antimicrobial chitosan–potato starch film by using microwave treatment followed: chitosan solution was prepared in 1% (v/v) glacial acetic acid solution. The prepared solution was stirred overnight at room temperature and filtered through synthetic cloth. Starch solution was prepared by dissolving starch powder in hot water. Chitosan solution was mixed with starch solution and then stirred at room temperature for a few hours. The final film-forming solution was poured into a petri dish. The films from chitosan and potato starch were successfully prepared by microwave treatment. Plasticizers were not used for making chitosan–potato starch film (Tripathi et al. 2009).

15.3.2 Preparation of Chitosan Film Enriched with Oregano Essential Oil (Chi et al. 2006)

Chitosan stock solution was prepared with 1.5% (w/w) chitosan in 1.5% v/v acetic acid. The solution was stirred overnight at room temperature, filtered through Miracloth®, and sterilized at 121°C for 15 min. At first, the essential oil was mixed with Tween® 20 and then added to the chitosan stock solution. The final film-forming solutions were homogenized under aseptic conditions at 21,600 rpm for 1 min and poured into sterile petri dishes. The films were dried under 5 psi vacuum at 30°C.

15.3.3 Preparation of Chitosan–Oleic Acid Edible Coatings (Vargas et al. 2006)

Chitosan (1%, w/v) was mixed in acetic acid solution (1%, v/v) at 40°C. Tween 80 at 0.1% (v/v) was added to improve wettability. The solution was stirred for 8 h and then oleic acid added to chitosan solution to reach a final concentration of 0%, 1%, 2%, and 4% (v/v). These mixtures were emulsified at 13,500 rpm for 4 min. In order to guarantee the stability of the emulsions, pH was adjusted to 5 with 1 N NaOH (Jumaa et al. 2002).

15.3.4 Preparation of Water-Soluble Chitosan and Amylose Film (Suzuki et al. 2005)

Aqueous amylose solution (1%) was prepared by dissolving amylose powder in hot water (80°C). A 1% salt free aqueous water-soluble chitosan (WSC) was obtained by the dialysis of a WSC solution that included NaCl produced by the neutralization of a dilute hydrochloric acid solution of WSC with NaOH. Each film having a thickness of 40–50 μm was prepared by casting the amylose, WSC, or their mixed solutions at 60°C. A film of fully deacetylated chitosan was obtained by casting a 0.1 M aqueous AcOH solution of fully deacetylated chitosan (1%) on a Kapton (polyimide) film. The resulting acidic chitosan film was neutralized with 1 M aqueous NaOH followed by washing with water and then dried.

15.3.5 Preparation of Chitosan Film Cross-Linked by Aglycone Geniposidic Acid (Mi et al. 2006)

Chitosan solution (1.5%, w/v) was prepared in 1.0% (v/v) acetic acid solution. The solution was allowed to stand overnight to remove air bubbles. Then the solution was poured into a glass disk in a dust-free environment and dried in air. The dried film was neutralized by 1 N NaOH solution and washed with phosphate-buffered saline (PBS). Aqueous aGSA solution was prepared. After that, chitosan film was immersed in aGSA solution for cross-linking. After 6 h, it was washed with deionized water to remove excess aGSA and dried in air.

15.3.6 Preparation of Glucomannan–Chitosan–Nisin Ternary Film (Li et al. 2006)

Konjac glucomannan was dissolved in distilled water and filtered out leaving a concentration of 1% (w/w). Chitosan dissolved in 0.8% (w/w) aqueous acetic acid to prepare 1% (w/w) solution. The solutions of konjac glucomannan/chitosan with different mixing ratios [9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 konjac glucomannan/chitosan (w/w) were cast onto polystyrene plates and dried at room temperature and then vacuum dried for 48 h to obtain the films. Nisin was incorporated into pure konjac glucomannan, chitosan film or the selected blend film forming solution at various levels to obtain antimicrobial films.

15.3.7 Preparation of Ferulic Acid Incorporated Starch–Chitosan Blend Film (Mathew and Abraham 2008)

Chitosan solutions (2%, w/v) prepared in 1% acetic acid solution and stirred overnight. After that, solutions were filtered with cheesecloth. Aqueous starch solutions (1%, w/v) were prepared by

heating, under stirring. A series of starch–chitosan blend films containing oxidized ferulic acid were prepared by mixing chitosan solution, starch solution, and different concentrations of ferulic acid (25, 50, 75, 100, and 200 mg/100 g of blend solution) in hydrogen peroxide and stirring for 1 h. Glycerol was added at 25% (w/w) of the total solid weight in solution. Then the solution was cast on to acrylic plates. After drying the films, they were peeled off from the plates and conditioned in desiccator for 48 h.

15.3.8 Preparation of Chitosan Films by Incorporating Garlic Oil, Potassium Sorbate, and Nisin (Pranoto et al. 2005)

Chitosan film was prepared by dissolving 1 g of shrimp chitosan in 100 mL of 1% acetic acid solution. The solution was filtered through a silk screen. The three antimicrobial agents—garlic oil, potassium sorbate, and nisin—were incorporated into chitosan film forming solution at various levels. The solutions were cast in polyacrylic plates and dried. The dry films obtained were peeled off and stored in a chamber at 50% RH and 25°C until evaluation.

15.3.9 Preparation of Antibacterial *O*-Carboxymethylated Chitosan/Cellulose Blend Film from LiCl/*N*,*N*-Dimethylacetamide Solution (Li et al. 2002)

Blend films I and II were prepared as follows.

Blend film I. 10 g O-CMCh was dissolved in 90 g de-ionized water. A certain amount of O-CMCh water solution was drip fed to cellulose solution under violent stirring. Then the mixture was spread on a glass plate. Finally, the films were dried at 50°C in an oven.

Blend film II. The pH of the O-CMCh water solution was adjusted to 14 with 1 M NaOH under vigorous agitation. The resulting O-CMCh water emulsion was washed with DMAc. The O-CMCh water emulsion was changed into O-CMChDMAc emulsion. Like O-CMCh water solution, a certain amount of the DMAc emulsion was also blended with cellulose solution and then films were regenerated in water.

15.3.10 Preparation of Chitin Whiskers by Using Supercritical Carbon Dioxide Treatment (Rinki et al. 2009)

Chitin (1 g) was treated in 3 N hydrochloric acid (100 mL) and stirred at 105°C for 3 h to obtain the colloidal solution. The residues were collected after centrifugation and were twice treated with hydrochloric acid. Finally, the residues were dialyzed in distilled water until they were neutral. The prepared chitin was then subjected to solvent exchange into acetone and ethyl alcohol prior to sc-CO_2 treatment.

15.3.11 Preparation of High Surface Area Chitosan (Rinki et al. 2009)

The supercritical carbon dioxide (sc-CO_2) treated chitin was treated in two ways: with NaOH and Na_2CO_3. 0.5 g of sc-CO_2-treated chitin was dissolved in aq. NaOH (40%, w/v, 50 mL) and stirred continuously for 7 h at 150°C and then left at room temperature overnight; then it was finally neutralized to obtain a crude product (1). For Na_2CO_3 alkaline treatment 0.5 g of sc-CO_2-treated chitin was dissolved in Na_2CO_3 (40%, w/v, 50 mL) and stirred continuously for 7 h at 90°C and left overnight at room temperature; finally it was also neutralized to get the crude product (2). The neutralization products (1 and 2) obtained after alkaline treatment were subjected to solvent exchange in acetone and ethyl alcohol prior to sc-CO_2 treatment to get the chitosan with high surface area.

15.4 EVALUATION AND TESTING METHODS OF ANTIMICROBIAL ACTIVITY

15.4.1 Evaluation of Antimicrobial Activity of Carboxymethyl Chitosan (CM), Quarternized Chitosan (Q), and Quarternized Carboxymethyl Chitosan (CMQ) In Vitro (Sun et al. 2005)

A series of quaternized carboxymethyl chitosan (CMQ), the sample no., and the characterization are listed in Table 15.1 (Sun et al. 2005). A Gram-positive bacterium *S. aureus* and a Gram-negative bacterium *E. coli* were used and inoculated on a gel containing 1% peptone, 2% agar, 3% meat extract, and 0.5% NaCl for this experiment.

The agar plate method was used to determine the minimum inhibition concentration (MIC) of CM, Q, and CMQ as follows: the samples were prepared at a concentration of 1% (w/v) and then autoclaved at 121°C for 25 min. Duplicate twofold serial dilutions of each sample were added to nutrient broth (beef extract 5 g, peptone 10 g to 1000 mL distilled water, pH 7.0) for final concentration of 0.1%, 0.05%, 0.025%, 0.0125%, 0.00625%, and 0.00313%. Some samples were prepared and diluted by the same way except for a final concentration of 0.00065% and 0.00033%. The culture of each bacterium was diluted by sterile distilled water to 105–106 CFU mL^{-1}. A loop of each suspension was inoculated on nutrient medium with sample or control added. After inoculation, the plates were incubated at 37°C for 72 h, the colonies were counted, and the MIC values were obtained. The MIC was considered to be the lowest concentration that completely inhibited against on agar plates comparing, disregarding a single colony or a faint haze caused by the inoculum (Speciale et al. 2002).

15.4.2 Antimicrobial Activity of CM and CMQ

The antimicrobial activities of CM, Q, and CMQ are shown in Tables 15.2 and 15.3. It was found that these samples showed effective antimicrobial activity against not only *E. coli* but also *S. aureus*, which were used in the test, although differences existed among them. Generally, the samples had more effective inhibition on *S. aureus* than *E. coli*. This difference may be attributed to their different cell walls. In *S. aureus,* a typical Gram-positive bacterium, its cell wall is fully composed of the peptide polyglycogen. The peptidoglycan layer is composed of networks with plenty of pores, which allow foreign molecules to come into the cell without difficulty. But the cell wall of *E. coli*, a typical Gram-negative bacterium, is made up of a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein, and phospholipids. Because of the bilayer structure, the outer membrane is a potential barrier against foreign molecules.

TABLE 15.1
Sample No. and Characterization of Different CMQ

Sample No.	DS of CM	DS of Q	Sample No.	M_w (×10^5)
CM_3Q_1	0.73	0.78		
CM_3Q_2	0.73	0.59	CM_3Q_2-1	4.72
CM_3Q_3	0.73	0.32	CM_3Q_2-2	2.28
CM_1Q_2	0.45	0.59	CM_3Q_2-3	0.45
CM_2Q_2	0.56	0.59	CM_3Q_2-4	0.11
CM_4Q_2	0.86	0.59		

Source: Sun, L. et al., *Asian Chitin J.*, 1, 39, 2005. With permission.

TABLE 15.2
Antimicrobial Activity of Chitosan (CS), Carboxymethyl Chitosan (CM), Quarternized Chitosan (Q), and Quarternized Carboxymethyl Chitosan (CMQ)

Samples	DS of CM	DS of Q	M_w ($\times 10^5$)	*E. coli*	*S. aureus*
CM	0.46	—	4.30	0.05	0.1
Q	—	0.60	3.89	0.0125	0.025
CMQ	0.45	0.59	4.51	0.00625	0.0125

Source: Sun, L. et al., *Asian Chitin J*., 1, 39, 2005. With permission.

TABLE 15.3
The Antimicrobial Activity of CMQ with Different Molecular Structure Factor

Samples	DS of CMC	DS of QC	M_w ($\times 10^5$)	*E. coli*	*S. aureus*
CM_1Q_2	0.45	0.59	4.51	0.00625	0.0125
CM_2Q_2	0.56	0.59	4.64	0.00625	0.0125
CM_3Q_2	0.73	0.59	4.72	0.00625	0.00625
CM_4Q_2	0.86	0.59	4.66	0.00625	0.0125
CM_3Q_1	0.73	0.78	4.21	0.0125	0.0125
CM_3Q_3	0.73	0.32	4.83	<0.00625	0.00625
CM_3Q_2-1	0.73	0.59	4.72	0.00625	0.0125
CM_3Q_2-2	0.73	0.59	2.28	0.00625	0.00625
CM_3Q_2-3	0.73	0.59	0.45	0.00313	0.00313
CM_3Q_2-4	0.73	0.59	0.11	<0.00313	0.00313

Source: Sun, L. et al., *Asian Chitin J*., 1, 39, 2005. With permission.

Compared with CM, Q, and CMQ in Table 15.3, CMQ had much better antimicrobial activities, whose MIC values were four to eight times lower than those of CM and two to four times lower than those of Q. It was noticed that the introduction of carboxymethyl group and quarternized group to the chitosan chain greatly enhanced the antimicrobial activity of the CMQ. We can deduce that carboxymethyl group and quaternary ammonium group act in a synergistic way.

As shown in Table 15.3, compared with CM_1Q_2, CM_2Q_2, CM_3Q_2, and CM_4Q_2, which have same degree of substitution of quaternized group, the authors found that no clear effect of DS value of carboxymethyl group on antimicrobial activity. Compared with CM_3Q_1, CM_3Q_2, and CM_3Q_3, which have same degree of substitution of carboxymethyl group, it can be observed that their antimicrobial activities are enhanced with increasing of the DS value. Compared with CM_3Q_2-1, CM_3Q_2-2, CM_3Q_2-3, and CM_3Q_2-4, which have same degree of substitution of both carboxymethyl group and quaternized group, the results demonstrated that their antimicrobial activity was affected by their molecular weight remarkably. Lower molecular weight resulted in better antimicrobial ability. When molecular weight was below 1×10^4, the antimicrobial activity of CMQ was strong and the MIC values reached to 0.00313%.

The antimicrobial mechanisms of CM, Q, and CMQ suggested to be as follows. On the one hand, the positive charge of the group at C-2 resulted in a polycationic structure, which can be expected to interact with the predominantly anionic components (lipopolysaccharides, proteins) on the surface of the microorganisms (Helander et al. 2000). The interaction resulted in great alteration of the structure

of outer membrane, which caused the release of a major proportion of proteinaceous material from the cells (Helander et al. 1998) when the quarternized group was introduced onto the molecular chain, the positive charge was strengthened. On the other hand, when carboxymethyl group was introduced along the molecular chain, the presence of a molecular with hydrophilic ends and forming weak interaction between hydrophilic ends and chitosan enhances the antimicrobial activity.

15.4.3 Antibacterial Assessment of Chitosan–Starch

The inhibitory effect of chitosan–starch solution and that of chitosan–starch film against *E. coli, S. aureus*, and *Bacillus subtilis* are shown in Figures 15.1a through c and 15.2a through c. The inhibitory effect was measured based on clear zone surrounding circular film strips/solution. The measurement of clear zone diameter included diameter of film strips/solutions; therefore, the values were always higher than the diameter of film strips/solutions whenever clearing zone was present. If there is no surrounding clear zone, we assumed that there is no inhibitory zone, and furthermore, the diameter was valued as zero. The results were observed and noted as follows (Table 15.4).

In terms of the surrounding clearing zone, chitosan–potato starch film did not show inhibitory effect against all tested microorganisms. The chitosan–starch film-forming solution showed a clear inhibitory zone of 1.5, 1.2, and 1.4 cm against *E. coli, S. aureus*, and *B. subtilis*, respectively. However, increasing level of starch at higher concentration did not reveal significantly increased inhibitory effect. It was generally caused by the maximum capability of chitosan polymer to carry active agents besides the occurrence of functional groups interaction phenomenon. The antimicrobial effect of chitosan took place without migration of active agents. As chitosan is in a solid form, therefore, only organisms in direct contact with the active sites of chitosan is inhibited. Chitosan

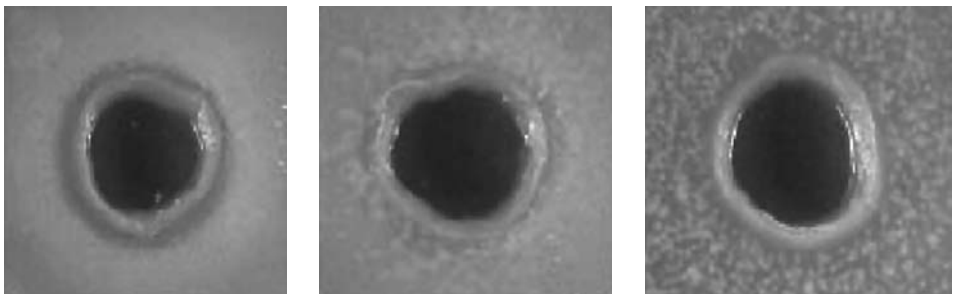


FIGURE 15.1 Inhibitory effect of chitosan–starch solution against (a) *E. coli*, (b) *S. aureus*, and (c) *B. subtilis*. (From Tripathi, S. et al., *Asian Chitin J.*, 4, 29, 2008a. With permission.)

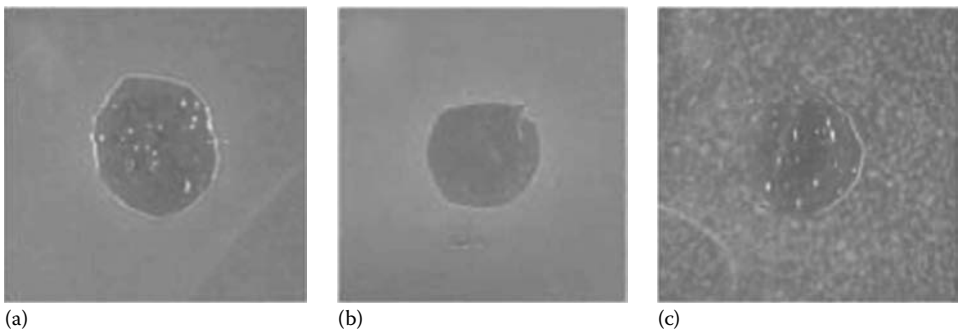


FIGURE 15.2 Inhibitory effect of chitosan–starch film against (a) *E. coli*, (b) *S. aureus*, and (c) *B. subtilis*. (From Tripathi, S. et al., *Asian Chitin J.*, 4, 29, 2008a. With permission.)

TABLE 15.4
Diameter of Inhibitory Zone of the Solution and Film against *E. coli*, *S. aureus*, and *B. subtilis*

	Diameter (cm) of Inhibitory Zone of	
Test Culture	**The Solution**	**The Film**
E. coli	1.5	0
S. aureus	1.2	0
B. subtilis	1.4	0

Source: Tripathi, S. et al., *Asian Chitin J.*, 4, 29, 2008a. With permission.

is incapable of diffusing through the adjacent agar media. The agar diffusion test is a method commonly used to examine antimicrobial activity regarding the diffusion of the compound tested through water-containing agar plate. The diffusion itself is dependent on the size, shape, and polarity of the diffusion material. The chemical structure and the crosslinking level of the films also affect this phenomenon. The chitosan–starch solution shows stronger inhibitory effect against *E. coli* and *B. subtilis* than *S. aureus*. Furthermore, it was found that the bioactive chitosan–potato starch film can be used to extend food shelf life (Tripathi et al. 2008a,b).

Incorporating chitosan and lauric acid into starch-based film showed more effective antimicrobial ability against *B. subtilis* and *E. coli* (Salleh et al. 2007). In this study, incorporating chitosan and lauric acid into starch-based film, obvious effects toward inhibition of *B. subtilis* and *E. coli* have been observed while the film had synergistic antimicrobial effect when chitosan and lauric acid were combined. Antimicrobial starch-based film incorporated with lauric acid and chitosan showed good flexibility than when purely starch-based film was formulated and formed (Figure 15.3). Inhibition of bacterial growth was examined using two methods, i.e., zone of inhibition test on solid media and liquid culture test (optical density measurements). The inhibitory activity was measured based on the diameter of the clear inhibition zone. The solution of starch and chitosan with different mixing ratios (w/w), that is 8:2 and 9:1, were the most effective mixing ratios, which had greater inhibition on both *B. subtilis* and *E. coli* than other solutions in agar plate and liquid culture test. The control (pure wheat starch) and AM film (incorporated with chitosan and lauric acid) were produced by casting method.

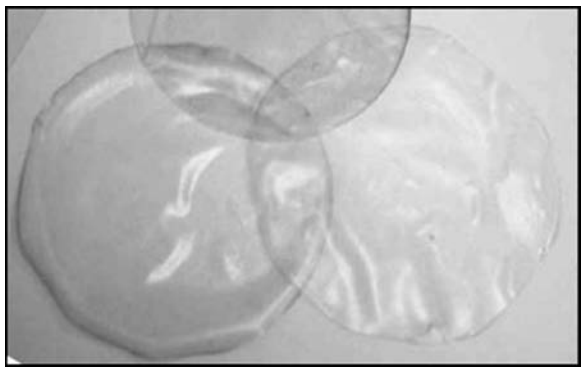
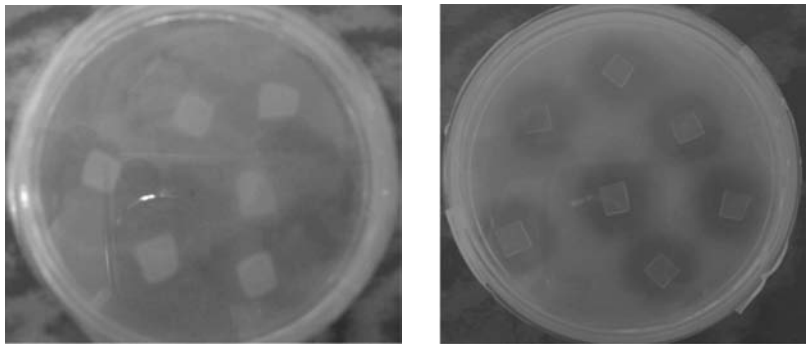


FIGURE 15.3 A translucent starch-based film incorporated with lauric acid and chitosan. (From Salleh, E. et al., *Asian Chitin J.*, 3, 55, 2007. With permission.)



(a) (b)

FIGURE 15.4 Inhibition area of (a) control film and (b) AM incorporated film. (From Salleh, E. et al., *Asian Chitin J.*, 3, 55, 2007. With permission.)

The antimicrobial effectiveness of control (pure wheat starch) and AM film incorporated with chitosan and lauric acid are shown in Figure 15.4a and b. A wide clear zone on solid media was observed for *B. subtilis* growth inhibition whereas inhibition for *E. coli* (*E. coli*) was not as effective as *B. subtilis*. From the liquid culture test, the AM films clearly demonstrated a better inhibition against *B. subtilis* than *E. coli*.

The tensile properties of the antimicrobial starch-based film had been improved by the addition of chitosan. These antimicrobial starch-based films can be used to extend food shelf life.

15.4.4 Antimicrobial Activity of Chitosan–PVA Film (Tripathi et al. 2009)

The inhibitory effect of chitosan–PVA solution and that of chitosan–PVA film against *E. coli, S. aureus*, and *B. subtilis* were studied by Tripathi et al. (2009). The inhibitory effect was measured based on clear zone surrounding circular film strips/solution. The measurement of clear zone diameter included diameter of film strips/solutions; therefore, the values were always higher than the diameter of film strips/solutions whenever clearing zone was present. If there is no surrounding clear zone, the authors assumed that there is no inhibitory zone, and furthermore, the diameter was valued as zero. In terms of surrounding clearing zone, chitosan–PVA film did not show inhibitory effect against all tested microorganisms. The chitosan–PVA film-forming solution showed a clear inhibitory zone against *E. coli, S. aureus* and *B. subtilis*, respectively. The chitosan–PVA solution shows stronger inhibitory effect against *E. coli* and *B. subtilis* than *S. aureus*. Furthermore, it was found that the bioactive chitosan–PVA film can be used to extend the shelf life of food. Chitosan-based antimicrobial films consisting of chitosan and PVA were prepared by solution casting method. These results pointed out that there is a molecular miscibility between PVA and chitosan. Chitosan-based antimicrobial film may be a promising material as a packaging film.

15.4.5 Antimicrobial Activity of sc-CO_2-Treated Chitosan-L-Glutamic Acid Aerogel Derivative (Singh et al. 2008)

The inhibitory activity of highly soluble sc-CO_2-treated chitosan-L-glutamic acid aerogel derivative (CL-GA) were studied against *E. coli*, *B. subtilis*, and *S. aureus*. The inhibitory activity was measured based on the diameter of clear inhibition zone surrounding sterilized disks. Whenever clear zone was present, the clear zone diameter was always higher than the diameter of sterilized disks in solution. If there was no clear zone surrounding, it was assumed that there was no inhibitory zone. CL-GA showed a clear inhibitory zone of 8 mm diameter against *B. subtilis* and *S. aureus*. Chitosan CL-GA did not show inhibitory effect against *E. coli*. Infact, one of the reasons for the antimicrobial

character of chitosan is its positively charged amino group which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms (Shahidi et al. 1999). Furthermore, it was found that CL-GA showed very small antimicrobial activity against *B. subtilis* and *S. aureus*. Further work of biological activity on CL-GA matrices is under progress.

15.4.6 Preservation of Vacuum-Packaged Processed Meats

The feasibility of improving the preservation of vacuum-packaged processed meats during refrigerated storage by use of an antimicrobial film designed to gradually release antimicrobial agents at the product surface (Ouattara et al. 2000a,b). The antimicrobial films were applied onto bologna, regular cooked ham or pastrami. The activity of the various films for inhibiting bacterial growth was tested against indigenous lactic acid bacteria and Enterobacteriaceae, and against *Lactobacillus sakei* or *Serratia liquefaciens*, surface-inoculated onto the meat products. The growth of Enterobacteriaceae and *S. liquefaciens* was delayed by application of the antimicrobial film. It was found that the inhibition of indigenous Enterobacteriaceae was more extensive at the surface of bologna than at the surface of pastrami, irrespective of film type. It is due to the fact that bologna contains efficient water-binding agents, and so exudes little water during storage.

15.4.7 Antibacterial Properties of Crawfish Chitosan (Nadarajah 2005)

15.4.7.1 Zone Inhibition Test

The antibacterial properties of crawfish chitosan films, made of different organic acids and chitosans, on selected food pathogenic bacteria: *L. monocytogenes*, *Bacillus cereus*, *Shigella sonnei*, *E. coli* (O157:H7), *S. aureus*, *S. typhimurium*, and *Vibrio vulnificus.*

The antibacterial activity of crawfish chitosan film formulations against seven pathogenic bacteria was expressed in terms of zone inhibition. The zone inhibition assay revealed primarily three types of observations, namely, defaced films without any clear or inhibition zones, which could be attributed to the absence of any inhibitory activity; clear zones without inhibitory zones, which could be attributed to bacteriostatic activity; and clear inhibition zone representing bacterialcidal inhibition by films.

15.4.7.2 Direct Inoculation Assay

The results of the direct inoculation study were in agreement with the inhibition zone assays (the survivor log number CFU mL^{-1} of *L. monocytogenes* inoculated onto the surface of selected chitosan films (DMCA acetate, DPMCA formate, and DMA citrate)). *L. monocytogenes* was more susceptible to chitosan citrate film than chitosan formate or chitosan acetate films. Chitosan citrate film reduced the bacterial count by 5.34 log CFU mL^{-1} within 4 h of incubation. Chitosan citrate films accounted for more than 4.47 log CFU mL^{-1} reduction of inoculum in 24 h. Chitosan acetate films caused only marginal reduction of the inoculum, accounting for less than 1 log CFU mL^{-1} reduction over the entire 24 h period incubation. The chitosan formate films caused about 1 log CFU mL^{-1} reduction of inoculum at 2 h of incubation and maintained a 1 log CFU mL^{-1} reduction over 24 h of incubation. The rate of reduction of microbial count was poor with both chitosan acetate and formate films as there was no significant difference in microbial count between 2 and 4 h incubation and between 4 and 8 h incubation. Organic acids with smaller molecular weight have higher antimicrobial activity and undissociated smaller molecules of formic (46.03 Da) and acetic (60.05 Da) acids may enter into the bacterial cells easily to change the internal pH of the organisms (Eswaranandam et al. 2004). Undissociated larger molecules of citric acid (192.13 Da) may not enter into the cells effectively. However, this trend was not observed in the above study and the result was in contrary. It indicates that chitosan films made of organic acids may behave as one entity rather than separate entities, i.e., as a career matrix

containing an antimicrobial agent. Several studies have demonstrated that antimicrobial edible films can reduce bacterial levels on meat products. Siragusa and Dickson (1992, 1993) showed that organic acids were more effective against *L. monocytogenes* on beef carcass tissue when immobilized in calcium alginate than when used as a spray or dip.

15.4.7.3 Bacterial Growth Susceptibility (Struszczyk and Pospieszny 1997)

Bacterial growth susceptibility was determined by the MIC method. Drops of chitin derivatives of different concentrations were applied to the surface of agarose plates containing cultures of bacteria in nutrient dextrose medium or LB medium for phytopathogenic bacteria and *E. coli*, respectively. MIC was defined as the lowest concentration of chitin derivatives that inhibited bacterial growth after overnight incubation of the agarose plates at 37°C.

In another experiment, the effect of chitin derivatives on *Pseudomonas syringae* pv. *phaseolicola* was tested using the hypersensitive reaction (HR) of tobacco. Mixtures of bacterium and chitin derivatives at a final concentration of 5×10^7 CFU mL^{-1} and 0.05 wt.%, respectively, were injected into leaves of tobacco *Xanthi nc*. The suspension of the bacterium in distilled water or solutions of chitin derivatives in distilled water was used as controls.

Water-soluble chitin oligomers, chitosan, chitosan sulfates, and carboxymethyl chitosan were used in this research. Chitosan was dissolved in the acetic acid and other chitin derivatives in distilled water. The reactions of all solutions were adjusted to pH of 5.5–6.0 with potassium hydroxide. Cationic chitin derivatives, i.e., chitin oligomers and chitosan, inhibited growth of the Gram-positive bacteria: *Clavibacter michiganense* subsp. *michiganense* and *C. michiganense* subsp. *insidiosum*, and Gram-negative bacteria*: Xanthomonas campestris* pv. *phaseoli*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. tomato, *Erwinia amylovora, Erwinia carotovora* subsp. *carotovora*, and *Agrobacterium tumefaciens* at a concentration of 0.01–0.3 wt%. However, both derivatives were less effective against *E. coli*. Anionic chitin derivatives, i.e., chitosan sulfate and carboxymethyl chitosan at a concentration of 1.5 wt% were not effective against any of the bacteria tested. When cationic derivatives were added to the bacteria suspension, their flocculation was observed. The hypersensitive reaction (HR) of plants is widely used for quick demonstration of bacterial pathogenicity (Klement 1963). When the tobacco leaves were injected by a mixture of *P. syringae* pv. *phaseolicola* and chitin derivatives, HR was prevented. A strong attachment of heterologous bacteria to the walls in tobacco leaves is essential to elicit the HR. Therefore, from mechanistic point of view, it is possible that chitin derivatives prevent the attachment of bacterial cells into the plant cell walls or affect their survival in the intercellular spaces.

15.4.7.4 Chemical Depolymerization

Chitosan oligosaccharides have received attention because of their versatile biological properties. They have lower viscosity, low molecular weights, and are soluble in aqueous solution. They seem to be readily adsorbed in vivo (Chatterjee et al. 2005).

The chemical treatment of chitosan using strong acids, e.g., HNO_2 and HCl, is a very common and fast method to produce a series of chitooligomers. However, this method has some disadvantages such as high cost, and the yield of chitosan oligosaccharides with degree of depolymerization (DP) from DP2 to DP5 is low because of random cleavage resulting in mostly monosaccharides. The irradiation effect on chitosan in acetic acid solution with various dose rates and the yield of chitosan oligomers were investigated (Chou et al. 2002). Low-molecular-weight chitosans were prepared at different reaction temperatures and time using 85% phosphoric acid that resulted in decrease of viscosity-average molecular weight from 21.4×10^4 to 7.1×10^4. Depolymerization of chitosan by the use of HNO_2 is a homogeneous reaction where the number of glycosidic bonds broken is stoichiometric to the amount of HNO_2 used (Jia and Shen 2002). The hydrolysis of chitosan with strong HCl was studied over a range of acid concentration and temperature. There have been very few reports on the degradation of chitosan by free radicals. Nordtveit et al. (1994) demonstrated that the viscosity of chitosan solution decreased rapidly in the presence of H_2O_2 and $FeCl_3$ probably due to random depolymerization of chitosan (Chen et al. 1997).

15.4.7.5 Enzymatic Depolymerization

Enzymatic depolymerization seems to be a better method to prepare chito oligosaccharides. Microorganisms have been found to possess chitosanase activity. Among bacteria, *Bacillus* and *Streptomyces* strains are most often studied. Studies on fungal chitosanase are less reported (Cheng and Li 2000).

The growing consumer demand for foods without chemical preservatives has resulted in the discovery of new natural antimicrobials (No et al. 2002). In this context, the antimicrobial activity of chitosan and their derivatives against different groups of microorganisms, such as bacteria, yeast, and fungi has received considerable attention in recent years. Antibacterial activities of six chitosans and six chitosan oligomers with different molecular weights were examined against four Gram-negative (*E. coli, Pseudomonas fluorescens, S. typhimurium*, and *Vibrio parahaemolyticus*) and seven Gram-positive bacteria (*L. monocytogenes*, *Bacillus mageterium*, *B. cereus*, *S. aureus*, *L. plantarum*, *Lactobacillus brevis*, and *Lactobacillus bulgaricus*). Chitosans showed higher antibacterial activities than chitosan oligomers and markedly inhibited growth of most bacteria tested although inhibitory effects differed with molecular weights of chitosan and the particular bacterium. Chitosan generally showed stronger bactericidal effects with Gram-positive bacteria than Gram-negative bacteria in the presence of 0.1% chitosan (Wang 1992). The MIC of chitosans ranged 0.05% to >0.1% depending on the bacteria and molecular weights of chitosan.

15.4.7.6 Chemical Modifications

Even though chitosan is quite attractive as a biopolymer with distinctive physicochemical properties and biological activities, it is currently utilized to only limited extent. The delay in application study is partly ascribable to the difficulty in controlled modifications because of the insoluble nature of organic solvents and multifunctionality of chitosan. However, many kinds of modification reactions have been exploited to increase the antimicrobial properties of chitosan. The growing demand for a more accurate control of polysaccharide properties has prompted the development of numerous techniques for selective modifications. The amino and two hydroxyl groups present in the repeating unit of chitosan are the targets of different chemical modifications (Hirano et al. 1987). As a result, the functionality of linear polysaccharides is significantly affected by the presence, level, and distribution of substituents along the main chain. As specified by Rabea et al. (2003), chitosan and chitin are commercially interesting compounds because of their high nitrogen content (6.89%) compared to synthetically substituted cellulose (1.25%). This makes chitosan a useful chelating agent. However, these naturally abundant materials are also limited in their reactivity and processability.

Several alkylated chitosans are reported to be synthesized. Kim et al. (1997a,b) prepared *N*-alkyl chitosan derivatives by introducing alkyl groups into the amine groups of chitosan via Schiff's base intermediates. Indeed, the free amine groups of the chitosan react with aldehydes to give the Schiff's base (Figure 15.5) in homogeneous medium such as acetic acid and methanol (Hirano 1997).

The antibacterial effects of the chemical modifications, long alkyl chains (until C12), the antibacterial activities of the quaternary ammonium salt of chitosan, and biocidal activity have also been studied and compared elsewhere.

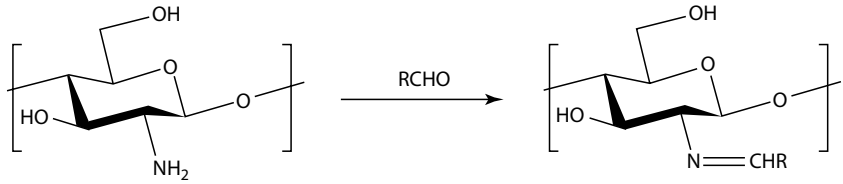


FIGURE 15.5 Shiff's base obtained from reaction between free amino groups of chitosan and aldehydes.

15.5 MECHANISM OF ANTIMICROBIAL ACTION

Because of the positive charge on the C-2 of the glucosamine monomer below pH 6, chitosan is more soluble and has a better antimicrobial activity than chitin (Chen et al. 1998). The exact mechanism of the antimicrobial action of chitin, chitosan, and their derivatives is still imperfectly known, but different mechanisms have been proposed (Rabea et al. 2003). One of the reasons for the antimicrobial character of chitosan is its positively charged amino group, which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms (Shahidi et al. 1999). Chitosan acted mainly on the outer surface of bacteria. At a lower concentration (0.2 mg mL^{-1}), polycationic chitosan does probably bind to the negatively charged bacterial surface to cause agglutination, while at higher concentrations, the larger number of positive charges may have imparted a net positive charge to the bacterial surfaces to keep them in suspension (Papineau et al. 1991; Sudarshan et al. 1992).

It has been postulated that the antimicrobial action of chitosan occurs as a result of several mechanisms. Chung and Chen (2008) studied the antibacterial activity of chitosan with respect to the extent of damaged or missing cell walls and the degree of leakage of enzymes and nucleotides from different cellular locations. First, the addition of chitosan to the bacterial suspension seemed to have a stronger impact on the Gram-negative *E. coli* than on the Gram-positive *S. aureus* in terms of the leakage of enzymes. The experimental results also revealed that the antibacterial action of chitosan not only involves a reaction with the cell wall of the bacteria but that it also may affect the structure of the phospholipid bilayer in the cell membrane, thereby changing the permeability of the cell membrane, resulting in the release of some of the cellular components. This action was further enhanced when chitosan with a high degree of deacetylation was used. To gain a better understanding of the mechanism by which chitosan functions as a bactericide, the cells were also subjected to a known antibiotic, which reacts with the anionic phosphate group of phospholipids in the cell membrane, destroying the cell membrane structure and affecting its permeability.

15.6 FACTORS AFFECTING ANTIMICROBIAL ACTIVITY

There are various intrinsic and extrinsic factors that affect the antimicrobial activity of chitosan. It has been demonstrated that lower molecular weight chitosan (of less than 10 kDa) has a greater antimicrobial activity than native chitosans (Uchida et al. 1989). Furthermore, a degree of polymerization of at least 7 is required; lower molecular weight fractions have little or no activity (Ralston et al. 1964, Uchida et al. 1989). Highly deacetylated chitosans are more antimicrobial than those with a higher proportion of acetylated amino groups due to increased solubility and higher charge density (Sekiguchi et al. 1994).

Lower pH increases the antimicrobial activity of chitosan for much the same reasons, in addition to the "hurdle effect" of inflicting acid stress on the target organisms (Rhoades and Rastall). The surrounding matrix is the greatest single influence on antimicrobial activity. Being cationic, chitosan has the potential to bind to many food components such as alginates, pectins, proteins, and inorganic polyelectrolytes such as polyphosphate (Kubota and Kikuchi 1998). Solubility can be decreased by using high concentrations of low molecular weight electrolytes such as sodium halides, sodium phosphate, and organic anions (Roberts 1992).

The effect of the molecular weight on some antibacterial and antifungal activities has been explored (Chen 1998). Chitosan with a molecular weight ranging from 10,000 to 100,000 have been found to be helpful in restraining the growth of bacteria. In addition, chitosan with an average molecular weight of 9300 was effective in restraining *E. coli*, whereas that with a molecular weight of 2200 helped in accelerating the growth (Tokura et al. 1994). Moreover, the antibacterial activity of chitosan is influenced by its degree of deacetylation, its concentration in solution, and the pH of the medium. Antibacterial activities were also found to be increasing in the order *N*,*O*-carboxymethylated chitosan, chitosan, and *O*-carboxymethylated chitosan (Liu et al. 2002).

In addition to the formation of gas-permeable films, chitosan has a dual function: (a) to direct the interference of fungal growth and (b) to activate several defense processes (Bai et al. 1988). These defense mechanisms include accumulation of chitinases, synthesis of proteinase inhibitors, and lignification and induction of callous synthesis (El et al. 2000). When applied on wounded wheat leaves, chitosan induced lignifications and consequently restricted the growth of nonpathogenic fungi in wheat. Chitosan inhibited the growth of *Aspergillus flavus* and aflatoxin production in liquid culture, preharvest maize, and groundnut, and it also enhanced phytoalexin production in germinating peanut (Cuero et al. 1991a,b). Chitosan has also been found to inhibit growth and toxin production by *Alternaria alternata* fungal species *lycopersici* in culture (Bhaskara et al. 1998, Dornenburg and Knorr 1997).

15.7 CONCLUSIONS

Chitin, chitosan, and oligosaccharides have offered itself as versatile and promising biodegradable polymers owing to its antimicrobial activity and nontoxicity leading to their extensive use over a wide range of applications. Among various applications of antimicrobial materials, packaging is the very promising system for the future improvement of food quality and preservation during processing and storage. Antimicrobial packaging can also be helpful in extending the food shelf life. The functional properties of chitin, chitosan, and oligosaccharides films can be improved when these are combined with other film-forming and coating materials. The understanding of the factors affecting the antimicrobial activity, mechanism of antimicrobial action of chitin, chitosan and their oligosaccharides would be an added advantage to use these materials in a better way.

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16 Anti-Inflammatory Activity of Chitin, Chitosan, and Their Derivatives

Moon-Moo Kim and Se-Kwon Kim

CONTENTS

16.1 ACUTE AND CHRONIC INFLAMMATION

Inflammation involving a wide variety of physiological and pathological processes is a kind of body's immune response against pathogens, toxic chemicals, or physical injury. Symptoms such as redness, heat, swelling, and pain are expressed in the inflammatory area that can arise when an injurious agent persists or when the immune system attacks its own tissue. It is largely divided into acute and chronic inflammation. Acute inflammation is a short-term normal response that usually leads to tissue repair by leukocytes such as macrophages, neutrophils, and eosinophils recruited into the damaged region, removing the cause of inflammation by the production of a number of inflammatory mediators. In contrast, chronic inflammation may progress from acute inflammation if the stimuli persist, causing own tissue damage (Drayton et al. 2006). Therefore, chronic inflammation is a long-term pathological response that involves tissue destruction due to the incompletion of tissue repair recovered by matrix metalloproteinases (MMPs) inhibitors (Hu et al. 2007). It is well known that the dysregulation of immune response can lead to chronic inflammatory diseases, and pharmacological intervention is necessary to attenuate cellular inflammatory pathways. Such prolonged inflammation ultimately results in several chronic diseases, such as periodontal disease, hepatitis, arthritis, gastritis, colitis, and atherosclerosis. The most important factor in chronic inflammation has been known to be the nuclear factor-kappa B (NF-κB) transcription factor that plays a critical role in regulating both innate and adaptive immune responses (Hayden and Ghosh 2004). NF-κB modulates inflammatory response through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) (Barnes and Karin 1997, Chen et al. 1999, Baldwin 2001). Current approaches to the treatment of inflammation rely on the inhibition of pro-inflammatory mediator production. In particular, COX-2, the key inducible enzyme responsible for producing prostanoids, and NF-κB can be attractive targets for developing anti-inflammatory medicines. Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed drug for the treatment

of many inflammatory diseases. However, their major disadvantage is the high incidence of gastric, renal, and hepatic adverse effects caused by the inhibition of prostaglandin (PG) synthesis. In recent years, anti-inflammatory agents have been focused on selective inhibitors of COX-2 without influencing the activity of constitutive COX-1 (Donnelly and Hawkey 1997). In recent years, it has been reported that chronic inflammation is associated with an increased risk of malignant transformation. Phagocytic leukocytes in chronic inflammatory process produce large amounts of reactive metabolites of oxygen and nitrogen that induce oxidative stress and lead to the oxidation of fatty acids and proteins in cell membranes, thus impairing their normal function. Moreover, DNA mutation indirectly induced by these reactive intermediates can be a major cause of malignant transformation and age-related diseases. In another aspect, inflammation is involved in wound healing progression, which triggers the recruitment of leukocytes into skin injury. Neutrophils penetrate from local blood vessels into endothelia, and monocytes migrate from blood into tissues and differentiate into macrophages that secrete cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1β, and interleukin-6 regulate the immune responses, further recruiting immune cells to the site of infection. In addition, COX-2, one of the enzymes responsible for the production of PG, mediates the inflammatory response. COX-2 inhibition might decrease scar collagen deposition after cutaneous injury (Willoughby and Tomlinson 1999). Transforming growth factors-β are also secreted by platelets, fibroblasts, and macrophages within the injury and are thought to act as attractants or inhibitors of keratinocyte, fibroblast, and inflammatory cell migration, in the upregulation of collagen synthesis and the modulation of matrix turnover via effects on MMPs and their inhibitors (Witte 1998).

During the last few years, our group has studied the effect of chitins, chitosans, and their derivatives on inflammation and MMPs. We found that chitin, chitosan, and their derivatives may be potential candidates capable of preventing or treating arthritis, hepatitis, nephritis, gastritis, colitis, periodontitis, metastasis, and wound related to chronic inflammation diseases by inhibiting the activity and expression of proinflammatory mediators and MMPs. Several substantial reports regarding their anti-inflammatory effect is described below.

16.2 EFFECT OF CHITIN ON INFLAMMATION

Although a number of studies have widely investigated the effects of chitin, chitosan, and their derivatives, a few anti-inflammatory activities have recently been published. Although the anti-inflammatory effects of chitin and its derivatives have been rarely reported, in recent years the data regarding them have been accumulating. Da Silva et al. (2009) proposed that chitin is a size-dependent regulator of innate immunity. In these studies, large chitin fragments were inert, while both intermediate-sized chitin (40–70 μm) and small chitin (SC; <40 μm, largely 2–10 μm) stimulated TNF elaboration in murine bronchoalveolar or peritoneal macrophages. In addition, chitin stimulated TLR2, dectin-1, and the mannose receptor, differentially activated NF-κB and spleen tyrosine kinase, and stimulated the production of pro- and anti-inflammatory cytokines. Ngo et al. (2009) demonstrated that chitin oligosaccharides (NA-COS; Mw 229.21–593.12 Da) produced by the acidic hydrolysis of crab chitin can inhibit myeloperoxidase (MPO) activity in human myeloid cells (HL-60) and the oxidation of DNA and protein in mouse macrophages (Raw 264.7). Furthermore, their direct radical scavenging effect by 20,70-dichlorofluorescein (DCF) intensity and intracellular glutathione (GSH) level were significantly increased in a time-dependent manner in the presence of NA-COS, which acts a potent antioxidant in live cells (Ngo et al. 2008a). They suggested a probability that chitin can have an anti-inflammatory activity through anti-oxidant effect. Shibata et al. (1997a,b) reported that mouse spleen cells produced IL-12, TNF-α, and IFN-γ when stimulated with phagocytosable chitin particles. Their results indicate that mannose receptor-mediated phagocytosis is highly associated with the production of IFN-γ-inducing signaling factors such as IL-12 and TNF-α. Chitin revealed immunopotentiating activity via the production of interferon-γ.

16.3 EFFECT OF CHITOSAN ON INFLAMMATION

Chitosan also has in vivo stimulatory effect on both nitric oxide production and chemotaxis, and modulates the peroxide production. Chitooligosaccharides (COS) enhanced the migration of the mouse peritoneal macrophages into inflammatory area (Okamoto et al. 2003, Mori et al. 2005, Moon et al. 2007). Yoon et al. (2007) reported that lipopolysaccharide (LPS)-stimulated TNF-α and IL-6 secretion was significantly inhibited in the presence of chitosan oligosaccharide in RAW 264.7 cells. These results suggested that chitosan oligosaccharide may have the anti-inflammatory effect via the stimulus of TNF-α in the LPS-stimulated inflammation.

In another study, chitosan was confirmed to partially inhibit the secretion of both IL-8 and TNF-α from mast cells, and it was proposed that water-soluble chitosan has the potential to reduce the allergic inflammatory response (Kim et al. 2005). Since mast cells are necessary for allergic reactions and have been implicated in a number of neuroinflammatory diseases, chitosan nutraceuticals may help to prevent or alleviate some of these complications.

The ability of chitosan to inhibit an inflammatory response could be exploited in the nutraceutical industry by those interested in providing anti-inflammatory foods for the prevention and alleviation of inflammatory diseases. Ueno et al. (2001) described that chitosan promotes phagocytosis and the production of osteopontin and leukotriene B by polymorphonuclear leukocytes, activities such as phagocytosis, and the production of interleukin-1, transforming growth factor b1 and platelet-derived growth factor by macrophages, and the production of interleukin-8 by fibroblasts, enhancing immune responses. Mendis et al. (2007) demonstrated that COS with below 1 kDa suppressed the generation of intracellular radical species in B16F1, murine melanoma cell line as well as the activation of NF-κB, suggesting the prevention of oxidative stress-related disease. Moreover, the induction of intracellular GSH level was increased in the presence of COS, which exhibited protective effect on oxidative damage of genomic DNA regardless of molecular weight. Under normal physiological conditions, MMPs are expressed at low concentrations but increase promptly when chronic inflammation occurs in tissues.

The effect of chitin, chitosan, and their derivatives on MMPs has been the object of a limited number of scientific articles so far. These enzymes are a family of secreted or transmembrane endopeptidases that share structural domains and degrade extracellular matrix components. Kim and Kim (2006) reported the inhibitory effect of COS with high solubility and without toxicity on the activation and expression of MMP-2 in primary human dermal fibroblasts (HDFs). The highest inhibitory effect being exerted by hydrolyzed chitosans with molecular weights as low as 3–5 kDa. The inhibition is caused by the decrease of the gene expression and transcriptional activity of MMP-2 via the AP-1 transcription factor. Therefore, the said chitosans may prevent and treat several health problems mediated by MMP-2 such as metastasis and wrinkle formation. It was proposed that the inhibitory effect might be explained by the effective chelating capacity of chitosan for Zn^{2+} as a cofactor in MMP-2 (Kim and Kim 2006). Atomic force microscopy revealed a direct molecular interaction between MMP-2 and chitosan. Affinity chromatography revealed a high binding specificity of MMP-2 to chitosan, and a colorimetric assay suggested a noncompetitive inhibition of MMP-2 by chitosan (Gorzelanny et al. 2007). COS have an in vitro stimulatory effect on the release of TNF-α and IL-1β in macrophages. Moreover, they could be engulfed by macrophages: Scatchard analysis of 2-aminoacridone-oligochitosan in macrophages indicated that its internalization was mediated by a specific receptor on macrophage membrane. COS internalization is mediated by a macrophage lectin receptor with mannose specificity. In fact, chitosan administered intravenously to mice become bound to macrophage plasma membrane mannose/glucose receptors that mediate their internalization (Shibata et al. 1997a,b). Chitosan has also in vivo stimulatory effect on both nitric oxide production and modulates the peroxide production (Okamoto et al. 2003, Mori et al. 2005). Chitosan oligosaccharides have a stimulatory effect on macrophages, and are chemo-attractants for neutrophils in vitro and in vivo, promoting chemiotaxis.

16.4 EFFECT OF CHITIN DERIVATIVES ON INFLAMMATION

Katsuyuki et al. (2003) synthesized three types of chitin derivatives such as phosphated chitin (P-chitin), phosphated–sulfated chitin (PSchitin), and sulfated chitin (S-chitin) and investigated their anti-inflammatory effect using a canine model of chitosan-induced pneumonia. After the simultaneous administration of chitosan and chitin derivatives, the hematological findings showed that only PS-chitin prevented the decrease in white blood cell count and decreased the generation of hemorrhage in lung specimens.

16.5 EFFECT OF CHITOSAN DERIVATIVES ON INFLAMMATION

COS have been shown to regulate various cellular and biological functions. However, the effect of COS on inflammatory responses of the cells remains unclear. Lin et al. (2007) examined the regulatory effect of highly *N*-acetylated COS (NACOS) on TNF-α-induced endothelial cell (EC) E-selectin expression, which is crucial for leukocyte recruitment. The pretreatment of ECs with NACOS inhibited not only the TNF-α-induced E-selectin expression in a dose- and time-dependent manner at the transcriptional level but also the TNF-α-induced JNK activation, suggesting that JNK was involved in the inhibitory effect of NACOS on TNF-α-induced E-selectin expression. Moreover, NACOS repressed the TNF-α-induced p65 and p50 mRNA expressions, indicating that it blocked the TNF-α-induced increases in the binding activity and in vivo promoter binding of NF-κB in ECs. They provided a molecular mechanism by which NACOS inhibit TNF-α-induced E-selectin expression in ECs, and a basis for using NACOS in pharmaceutical therapy against inflammation. The cellular antioxidant effects of carboxylated COS (CCOS) shown in Figure 16.1 inhibited the oxidation of cellular biomolecules such as lipids, proteins, and exhibited direct scavenging of reactive oxygen species (ROS) in mouse macrophages, RAW264.7 cells.

Further, CCOS inhibited MPO activity in human myeloid cells (HL60), suggesting indirect possibility of inhibiting the generation of ROS such as superoxide radicals, H_2O_2, and HOCl (Rajapakse et al. 2007). After treatment with various concentrations of carboxymethyl chitosan (50, 100, 200 μg/mL), the following parameters were measured: apoptotic rate, mitochondrial function, nitric oxide production, levels of iNOS mRNA and ROS. The results showed that carboxymethyl chitosan could inhibit chondrocyte apoptosis in a dose-dependent manner. Furthermore, it could partly restore the levels of mitochondrial membrane potential and ATP, decrease nitric oxide production by downregulating nitric oxide synthase mRNA expression, and scavenge ROS in chondrocytes. The inhibitory effects of carboxymethyl chitosan on IL-1β-induced chondrocyte apoptosis were possibly due to the protection of the mitochondrial function, and the decline of nitric oxide and ROS (Chen et al. 2006). In addition, aminoethyl COS (AE-COS) shown in Figure 16.2 revealed the competitive inhibition on Angiostensin I converting enzyme that plays an important physiological role in regulating blood pressure by converting an inactive form of decapeptide, angiotensin I, to

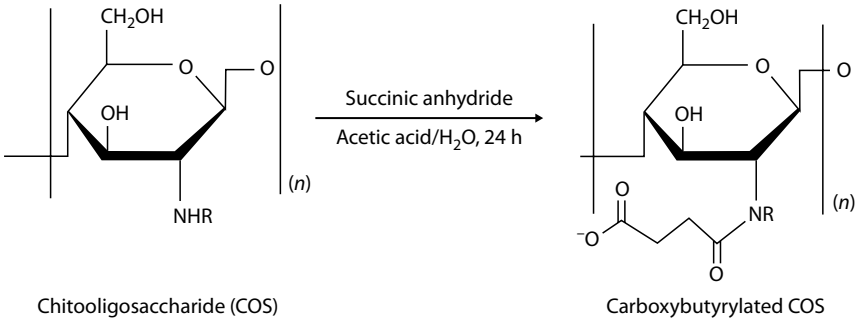

FIGURE 16.1 Schematic diagram for the synthesis of carboxybutyrylated chitgooligosaccharide.

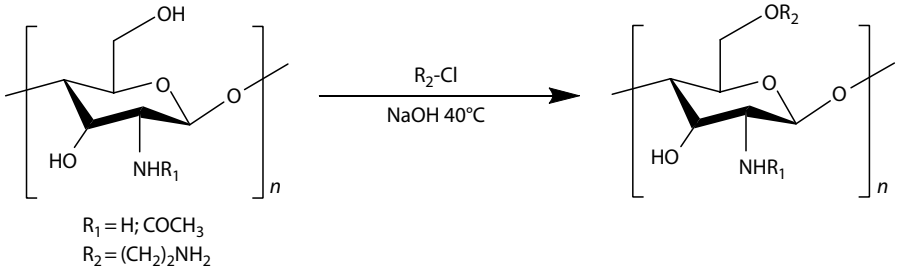

FIGURE 16.2 Synthesis of AE-COS.

a potent vasoconstrictor, octapeptide, angiotensin II, and by inactivating the catalytic function of bradykinin (Ngo et al. 2008b).

16.6 EFFECT OF GLUCOSAMINE AND ITS DERIVATIVES ON INFLAMMATION

The signal transduction pathway involved in the glucosamine (Glc) influence on the gene expression of MMPs was investigated in chondrocytes stimulated with IL-1β. Glc inhibited the expression and the synthesis of MMP-3 induced by IL-1β, and that inhibition was mediated at the level of transcription. The inhibition of the p38 pathway in the presence of Glc substantially explains the chondroprotective effect of Glc on chondrocytes that regulate COX-2 expression, PGE(2) synthesis, and NO expression and synthesis (Lin et al. 2007). A novel glucosamine (GlcN) derivative, carboxybutyrylated GlcN (CGlcN), inhibited the expression of iNOS and COX-2 in bacterial LPS-induced mouse macrophages (Rajapakse et al. 2008). They supported that the inhibition of iNOS and COX-2 is caused by the downregulation of p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK) via NF-κB. Moreover, CGlcN directly inhibits the degradation of IκB and release of NF-κB. Mendis et al. (2008) reported that quaternized amino Glc (QAGlc) shown in Figure 16.3, a cationic Glc derivative, inhibits LPS-stimulated production of IL-1β, IL-6, TNF-a, and PGE2 in RAW264.7 more effectively than Glc.

It exerted anti-inflammatory effect via the inhibition of NF-κB and regulation of MAPKs pathway such as p38 and JNK. Although, several effects of Glc and its sulfated form (sulfated Glc) have been proposed for the suppression of osteoarthritis (OA), their exact mechanisms have not been completely elucidated. Glc sulfate (SGlc) has been known to be effective in controlling OA symptoms by the mechanisms that SGlc can increase ALP activity, collagen synthesis, osteocalcin secretion, and mineralization in osteoblastic cells in vitro (Kim et al. 2007). Furthermore, it was observed that SGlc exhibited anti-inflammatory effect on the production of TNF-α, IL-1β, and PGE_2 in macrophage, RAW264.7 cells. In addition, SGlc has been reported to relieve joint pain and inflammation in many arthritis patients. It was demonstrated that SGlc as a potent MMP inhibitor exhibited the inhibition of MMP-2 and MMP-9 via the downregulation of the transcription factor,

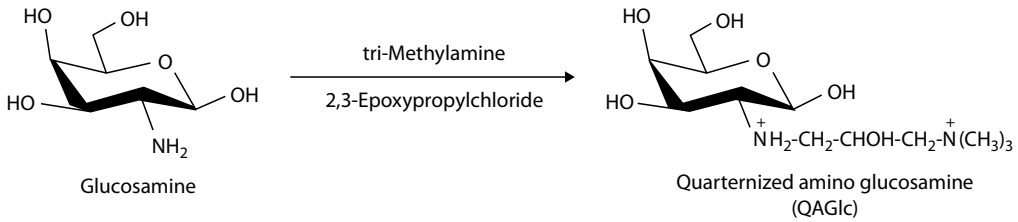

FIGURE 16.3 Synthesis of QAGIc was carried out with 2,3-epoxypropyl chloride and tri-methyl amine under controlled conditions.

NF-κB (Rajapakse et al. 2007). Mendis et al. (2008) described the novel possibility of the involvement of sulfated Glc in improving cellular antioxidant potential and thereby controlling oxidative damage. Treatment with sulfated Glc to human chondrocytes and macrophages inhibited the radical-simulated oxidation of membrane lipids, proteins, and DNA in a dose-dependent manner. Moreover, it was reported that sulfated Glc inhibits the radical-mediated expression and activation of NF-κB involved in the expression of a number of genes related to OA. Further, sulfated Glc enhanced reduced GSH level in oxidatively stressed human chondrocytes improving cellular redox balance. In conclusion, it is suggested that potential effects of sulfated Glc in controlling OA might be partly via mechanisms involving direct scavenging of cellular radical species and the alteration of oxidation-mediated destructive events.

16.7 CONCLUSIONS

Although a number of studies have widely investigated the effects of chitin, chitosan, and their derivatives, a few anti-inflammatory activities have recently been published. Although the anti-inflammatory effects of chitin and its derivatives have been rarely reported, in recent years chitin and chitosan derivatives as well as Glc derivatives exert anti-inflammatory effects via MAPK signaling transduction pathway regulated by NF-κB and AP-1 transcription factors that play a critical role in acute and chronic inflammation. Especially, some of them inhibit MMPs that are highly expressed and activated in chronic inflammation. Therefore, some of chitin, chitosan, and their derivatives with anti-inflammatory effect as well as low toxicity can prove to be potential candidates in developing functional foods and medication capable of preventing or treating several chronic diseases such as periodontal disease, hepatitis, arthritis, gastritis, colitis, and atherosclerosis.

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17 Chitosan Scaffolds for Bone Regeneration

Riccardo A. A. Muzzarelli

CONTENTS

17.1 INTRODUCTION

The chitins and chitosans of various origins and some of their derivatives obtained by chemical or enzymatic means are today protagonists in the scenario of wound healing, tissue engineering, and gene therapy, thanks to their outstanding properties. Basic information on these polysaccharides, relevant to the title topic, can be found in books and review articles (Varlamov et al. 2003, Kumar et al. 2004, Hollister 2005, Kurita 2006, Rinaudo 2006a,b, Uragami and Tokura 2006, Castagnino et al. 2008, Keong and Halin 2009, Yuan et al. 2009, Muzzarelli and Muzzarelli 2009, Muzzarelli 2009a,b,c, 2010).

17.1.1 Early Studies with Chitins and Chitosans

The ability of chitosan to bind to anionic compounds such as growth factors, glycosaminoglycans, and DNA is of great importance. In fact, the good biocompatibility, the intrinsic antibacterial activity, the recognition of growth factors, and the processability in a variety of different macro- and nanoshapes makes chitosan a prominent scaffold material for cartilage, intervertebral disc, and bone tissue substitutes. Moreover, the formation of chitosan–DNA complexes renders chitosan a good support in orthopedic gene therapy.

Certain studies dealing with the reconstruction of the periodontal tissue with chitosan were a prelude to the discovery of the osteoinductive properties of chitosan (Muzzarelli et al. 1989). Surgical wounds from wisdom tooth avulsions were treated with freeze-dried methylpyrrolidinone chitosan, which promoted bone regeneration. The polysaccharide was depolymerized by lysozyme and was no longer detectable 6 months after surgery. Methylpyrrolidinone chitosan was found to be useful in apicectomy as well: in fact, radiographic evidence of bone regeneration was obtained in human

patients undergoing apicectomies and avulsions. None of the patients reported adverse effects over 3 years of observation (Muzzarelli et al. 1993a). The existence of osteoprogenitor cells in a wound site with healthy tissues, such as the avulsion site, offers the possibility of regenerating the periodontal, peri-implant, and alveolar ridge bone tissue simply with the aid of chemical mediators from chitosan: the amount of bone-forming colonies is almost doubled in the presence of chitosan. In fact, chitosan stimulates the differentiation of osteoprogenitor cells and thereby facilitates the formation of bone (Klokkevold et al. 1996).

Chitosan was used to assist the spontaneous but difficult repair of the meniscus because it is well tolerated by the synovia; it favored and stimulated repair processes that do not take place spontaneously in the meniscus. Its initial angiogenetic action appeared to be effective enough to provide the meniscus with the necessary tissue components and humoral factors (Muzzarelli et al. 1992).

As a continuation of these early observations, bone defects surgically produced in sheep and rabbit models were treated with freeze-dried methylpyrrolidinone chitosan (Muzzarelli et al. 1993b, 1994, Mattioli-Belmonte et al. 1995). Histological examination performed 60 days after surgery showed a considerable presence of neo-formed bone tissue as compared to control. Endosteal, periosteal, and bone marrow osteoblast-like precursors, stimulated by growth factor entrapped in the polysaccharide, generated intramembranous bone formation. Bone osteoid formation was followed by mineralization. Osteoinduction was also observed in rabbit endochondral bones (Borah et al. 1992).

Studies on rabbit and sheep were performed in order to improve bone tissue reconstitution with chitosan associated with calcium phosphate. Microscopic and histological analyses showed the presence of an osteogenic reaction moving from the rim of the surgical lesion toward the center. In control lesions, dense fibrous tissue without the characteristic histoarchitecture of bone was observed. The pattern of bone regeneration was studied in an osteoporotic experimental model with the bone morphogenetic protein linked to the chitosan, whose degradation led to the release of the protein: as a consequence, the bone tissue regeneration in surgical bone defects was improved using the said combination. This important result also proved the validity of a biochemical approach to the therapy of various affections in the elderly (Muzzarelli et al. 1997).

Dicarboxymethyl chitosan and 6-oxychitin sodium salt applied to femoral surgical defects for 3 weeks produced a good histoarchitectural order in the newly formed bone tissue (Mattioli-Belmonte et al. 1999). Studies were carried out on the effects of dicarboxymethyl chitosan on the precipitation of a number of insoluble salts, including calcium phosphate (Muzzarelli et al. 1998). The chelating ability of this modified chitosan interfered with the physicochemical behavior of magnesium and calcium salts. Dicarboxymethyl chitosan mixed with calcium acetate and disodium hydrogen phosphate in suitable ratios yielded clear solutions from which an amorphous material (ca. 50% inorganic) was isolated and used for the treatment of bone lesions in experimental surgery and dentistry. Bone tissue regeneration was promoted in sheep, leading to complete healing of otherwise nonhealing surgical defects. The dicarboxymethyl chitosan + calcium phosphate chelate favored osteogenesis while promoting bone mineralization. The in situ precipitation route toward obtaining composites of polymer and calcium phosphate is similar to the strategy employed in naturally occurring biocomposites and proved to be a viable method for the synthesis of bone substitutes (Muzzarelli and Muzzarelli 2002).

Oxychitin, a modified chitin obtained via the oxidation of chitin at C6 and constituted by relatively short β(1–4) chains of 2-acetamido-2-deoxy glucuronic acid sodium salt, is functionally similar to hyaluronan and is endowed with anionic character and chelating ability. For the preparation of 6-oxychitin, the stable nitroxyl radical 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo®) was used as a catalyst, together with NaBr, to regiospecifically oxidize chitin with aqueous NaOCl. Oxychitin was fully characterized from the chemical and enzymatic standpoints and was sterilized by γ-ray irradiation at 25 kGy; the aspect was that of a white, soft, spongy, and water-soluble material. The average molecular weight of 6-oxychitin was close to 10 kDa, and the degree of substitution was 1.0.

Oxychitin, applied to femoral surgical defects for 3 weeks, produced a good histoarchitectural order in the newly formed bone tissue. When osteoblasts from newborn mouse calvariae were associated to the polysaccharide, these preparations showed enhanced tissue mimicking capacity. The spongious trabecular architecture was restored in the defect site and the association of the chitin derivative with the osteoblasts seemed to be one of the best biomaterials in terms of bone tissue recovery; the addition of osteoblasts improved the performance of 6-oxychitin and the spongious trabecular architecture of the newly formed bone was superior to other remedies. Oxychitin therefore represented an advance in the experimental study of the osteoinduction process and anticipated applications intended to reconstruct the correct morphology of bone tissues, even in the presence of important mechanical stress (Muzzarelli et al. 1999, 2000, 2001).

17.2 BONE SUBSTITUTES AND CEMENTS

The use of degradable polymers as matrices forms a major approach to the development of bone composites when compared to the nondegradable systems. Degradable matrices maximize the osteoconductive behavior of hydroxyapatite, allowing bone ingrowth into the implant to occur as the matrix is resorbed with time. In this respect, the degradable matrices act as binders to prevent the migration of hydroxyapatite from the implant site. The chitosan-bonded hydroxyapatite bone-filling paste was made as follows: chitosan (0.5 g) was dissolved in malic acid (0.5 g) solution made with saline, and a chitosan film was formed by mixing this solution with hydroxyapatite powder (2 g), followed by neutralization with 5% sodium polyphosphate. To help cells and blood vessels to penetrate this material, the tensile strength and elongation were optimized (Ito 1991, Maruyama and Ito 1996, Ito and Hidaka 1997). Similarly, a composite of hydroxyapatite and a network formed via cross-linking of chitosan and gelatin was made with glutaraldehyde (Yin et al. 2000).

The presence of hydroxyapatite did not retard the formation of the chitosan/gelatin network. Calcium phosphate cements are suitable for the repair and reconstruction of bone because after implantation in bone defects they are rapidly integrated into the bone structure, after which they are transformed into new bone thanks to the activity of osteoclasts and osteoblasts. These cements have the advantage that they can be molded during the operation and are injectable, that is, they adapt immediately to the bone cavity and permit subsequent good osteointegration. To make the cement injectable, several additives can be incorporated; however, the properties of the cement should be preserved: setting times suited to a convenient delay with surgical intervention, limited disintegration in aqueous medium, and sufficient mechanical resistance. Lactic acid, glycerol, glycerophosphate, and chitosan are adjuvants in terms of injectability, setting time, disintegration, and toughness (Leroux et al. 1999). Chitosan alone improved injectability, increased setting time, and limited the evolution of the cement toward hydroxyapatite by maintaining the octacalcium phosphate phase; therefore, only slight disintegration was observed. Because octacalcium phosphate is considered one of the precursors in bone tissue formation, the adsorption of chitosan on this salt was considered advantageous (Viala et al. 1996, 1998).

When a chitosan–hydroxyapatite paste was applied to the surface of the tibia after periosteum removal, the formation of new bone was observed after a week: it continued during a 20 week follow-up indicating suitability of this paste as a bone-filling material (Kawakami et al. 1992). The issue of mechanical resistance of chitosan-based composites was addressed by Hu et al. (2003) who reported a chitosan–hydroxyapatite multilayer nanocomposite with high strength and bending modulus suitable for the fixation of long bone fractures. A macroporous chitosan + beta-tricalcium phosphate composite scaffold for bone tissue engineering was developed by freeze-drying, and the effects of the composite concentration and of the freezing temperature on the ability to resist compression by the scaffold were studied (Leroux et al. 1999, Yin et al. 2003). Because chitosan solutions gelify in response to a pH change from slightly acidic to neutral, the chitosan + calcium phosphate composites satisfy the need to develop bone fillers that set in response to physiological conditions, but not while mixing the components in vitro.

The preparation of bone fillers evolved over the last decade: a recently proposed injectable bone substitute consisting of citric acid, chitosan solution as the liquid phase and tetracalcium phosphate, dicalcium phosphate anhydrous, and calcium sulfate hemihydrate powders as the solid phase was prepared. Four groups containing up to 30% of $CaSO_4 \cdot 0.5H_2O$ showed that the setting time for all compositions was in the range of 25–45 min, and that the injectability was improved by the addition of this salt. The enhancement of crystallinity was confirmed by SEM and XRD spectrometry: the XRD peak intensity of hydroxyapatite increased with time and quantity of $CaSO_4$. The maximum compressive strength obtained for the bone substitute was with 20% $CaSO_4$ after 28 day incubation in 100% humidity at 37°C (Song et al. 2009).

Because chitosan is interesting as a coating for dental, craniofacial, and orthopaedic implants, it was used by Wang et al. (2008) to increase the biocompatibility of electrolytically deposited apatite coatings on titanium alloys: that coating exhibited an improved bone marrow stromal cell attachment. Similar data were obtained for chitosan coupled with surface-immobilized cell-adhesive arginine–glycine–aspartic acid (RGD) peptide, and for hyaluronan by Chua et al. (2008). Likewise, titanium was coated with three chitosans of different degrees of deacetylation and from different manufacturers via silane + glutaraldehyde. Coating bond strength was in the range 2.2–3.8 MPa regardless of the degree of deacetylation. The coatings exhibited little dissolution over 5 weeks even in the presence of lysozyme, and were judged to be osteocompatible in vitro (Yuan et al. 2008). Cathodic electrophoretic deposition on stainless steel was utilized for the fabrication of composite hydroxyapatite–chitosan coatings (up to 60 μm thick). The coatings, whose composition was changed by altering the chitosan–hydroxyapatite ratio in the solutions, provided protection against corrosion (Pang and Zhitomirsky 2007). The use of an antibiotic-loaded chitosan coating on stainless steel screws in contaminated bone fracture fixation was considered after the optimization of antibiotic loading and release (Greene et al. 2008).

17.3 OSTEOGENIC CHITOSAN COMPOSITES

The cationic nature of chitosan is primarily responsible for electrostatic interactions with anionic glycosaminoglycans, proteoglycans, and other negatively charged molecules. A large number of cytokines/growth factors are linked to glycosaminoglycans (especially heparin and heparan sulphate), and a scaffold incorporating a chitosan–glycosaminoglycan complex may retain and concentrate growth factors most efficiently. For example, the layer-by-layer technique was used to assemble heparin (a strong polyanion) and chitosan: the polyelectrolyte complexes were characterized in terms of the sensitivity of the polyelectrolyte composition and layer thickness to changes of processing parameters (Boddohi et al. 2008).

Carbohydrate polymers exert a variety of biological actions in modulating the intra- and extracellular environment. Substituted dextrans bind growth factors and protect them from enzymatic degradation. Heparin-like dextrans enhance the healing of bone in an environment where bone would otherwise not regenerate (Albo et al. 1996). The binding of heparin-like polysaccharides to fibroblast growth factor (FGF) induces a conformational change in the latter, resulting in the formation of FGF dimers or oligomers, and this biologically active form becomes available to the FGF receptor for signal transduction (Venkataraman et al. 1996). Osteoinduction of the BMP–chitin complex was accompanied by excellent biocompatibility (Miyazawa 1995).

Interactions with extracellular matrix provide cells with information essential for controlling morphogenesis, migration, repair, and death (Werb 1997). Chitosan has been combined with a variety of materials such as alginate, hydroxyapatite, calcium phosphate, poly(methylmetacrylate), poly(L-lactic acid), and growth factors for potential application in orthopedics; hyaluronan showed morphogenetic activities suitable for a correct bone architecture. Chitosan improved cell adhesion, proliferation, biosynthetic activity, and chondrocyte attachment to poly (L-lactic acid) and alginate (Cui et al. 2003). The increase of extracellular Ca^{2+} is perceived by osteoblasts via specific receptors that lead to mutagenic and chemiotactic action; thus chitosans carrying calcium phosphate

accelerate bone wound healing. Bone defects surgically produced in sheep and rabbit models have been treated with freeze-dried modified chitosans because chitosan promotes direct endochondral ossification. Moreover, the pattern of bone regeneration has been studied in an osteoporotic experimental model with bone morphogenetic protein linked to chitosan (Muzzarelli et al. 1998). The chitosan + collagen scaffolds (scarcely deacetylated chitosan) had a significantly higher proliferative effect and alkaline phosphatase activity than scaffolds made of highly deacetylated chitosan, regardless of molecular weight. Scanning electron images demonstrated that MC3T3-E1 osteoblasts grew well on all test scaffolds; on the other hand, mineralized nodule formation was not found. The degree of deacetylation of chitosan is a crucial factor for osteoblasts, and it should be considered in bone tissue regeneration (Suphasiriroj et al. 2009).

Rat calvarial osteoblasts were grown in porous chitosan sponges fabricated by freeze-drying. The prepared chitosan sponges had a porous structure with a 100–200 μm pore diameter, which allowed cell proliferation. Cell density, alkaline phosphatase activity, and calcium deposition were monitored for up to 56 days. Cell numbers per gram of sponge were 4×10^6 (day 1), 11×10^6 (day 28) and 12×10^6 (day 56); calcium deposition was 9 (day 1), 40 (day 28), and 48 (day 56) μg/g of sponge. Histological results corroborated the observed bone formation within the sponges (Seol et al. 2004).

Chitosan promoted growth and mineral-rich matrix deposition by osteoblasts in culture and allowed osteoconduction (Muzzarelli et al. 1993a,b). In further studies, a three-dimensional macroporous calcium phosphate bioceramic embedded with porous chitosan was developed: in this scaffold, a nested chitosan sponge enhanced the mechanical strength of the ceramic component via matrix reinforcement, and preserved the osteoblast phenotype (Zhang and Zhang 2001, 2002a,b). Similarly, gentamycin-conjugated macroporous chitosan scaffolds reinforced with beta-tricalcium phosphate and calcium phosphate have been developed for bone engineering. Macroporous chitosan scaffolds incorporating hydroxyapatite or calcium phosphate glass with an interconnected porosity of approximately 100 5m have been synthesized (Zhang et al. 2003).

17.4 CHITOSANS WITH HYDROXYAPATITE

To meet the challenge of regenerating bone lost to disease or trauma, biodegradable scaffolds are being investigated as a way to regenerate bone without the need for auto- or allografts. As noted above, various forms of calcium phosphate have been proposed, and it was noted that hydroxyapatite and chitosan exert various actions on each other. In fact, the long-term aging of hydroxyapatite in chitosan acetate gel solutions resulted in changes in the surface chemistry, colloid stability, and the chemical composition of hydroxyapatite due to adsorption and solubility effects. Chitosan exhibited strong adsorption on hydroxyapatite particles and improved the colloid stability of hydroxyapatite. The amount of chitosan adsorbed on hydroxyapatite was 2.8%–3.1% based on elemental analysis. The specific surface area of hydroxyapatite doubled after aging in chitosan acetate solutions and attained 160 m^2/g (Wilson and Hull 2008). As an alternative, finely dispersed hydroxyapatite in chitin solution (LiCl–dimethylacetamide solvent) was stirred for 4 days at 10°C and cast into plastic molds to form hydroxyapatite + chitin gels: they were immersed in water for 1 week to remove residual LiCl and dimethylacetamide. The hydrogels were freeze-dried or air-dried to yield films.

Microscale hydroxyapatite (size distribution of the powder 3.0 ± 0.1 μm) similar to the innate hydroxyapatite in human bone (57.0 and 41.4 wt% of CaO and P_2O_5, respectively; Ca/P molar ratio 1.67) was used to fabricate films after suspension in solutions of 400 kDa chitosan. Because of the well-dispersed hydroxyapatite powders in the polymer matrix, the film appeared white and flexible with a transmittance <20% in the visible range. The observation under the SEM ensured monodispersity of the films. Good monodispersity improved the homogeneity and uniformity of the film, and prevented phase separation resulting in unwanted microcracks (Kim et al. 2009).

The chitosan + hydroxyapatite composite was also prepared in the form of membranes by coprecipitation, filtration, and freeze-drying. Needle-like hydroxyapatite nanocrystals with low

crystallinity were uniformly embedded in the chitosan matrix. As the hydroxyapatite content was increased, the tensile strength of the membranes exhibited a steady decrease, while the elastic modulus doubled when 20% hydroxyapatite was present. The results of the in vitro cell culture showed that the highest alkaline phosphatase level was achieved when 30% hydroxyapatite was present in the composites (Teng et al. 2009).

Chitosan derivatives are also suitable for hydroxyapatite composites: a composite scaffold made of crystalline hydroxyapatite and carboxymethyl chitosan was obtained using a coprecipitation method. The pores of the scaffold were regular, interconnected, with size in the range of 20–500 μm, characteristic diffraction peaks of apatite, and the presence of typical bands from carboxymethyl chitosan, thus showing that the coprecipitation of both organic and inorganic ingredients was effective. The composite scaffolds were found to possess a 58.9% ± 6% of porosity, and consisted of 24% hydroxyapatite and 76% carboxymethyl chitosan. The hydroxyapatite/carboxymethyl chitosan composite scaffolds showed degradability and bioactivity (Oliveira et al. 2009a).

17.4.1 Composites Containing Nano-Hydroxyapatite

An instrumental characterization of nano-hydroxyapatite was provided by Chen et al. (2002): the size of their particles was ca. 20–30 nm in width and 50–60 nm in length, with a specific surface area of 73 m^2/g; aggregation was avoided with the use of *n*-butanol. The uniform dispersion of these particles in chitosan solutions was recognized as an important motif for the innovative preparation of chitosan composites: in fact, nano-hydroxyapatite + chitosan composite membranes prepared by solvent casting and evaporation were found suitable for guided bone regeneration.

The nano-hydroxyapatite was prepared according to the following chemical equation:

$$Ca(NO_3)_2 \cdot 4H_2O + (NH_4)_3PO_4 \cdot 3H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2$$

While the pH value was kept above 10 with ammonium hydroxide, the precipitated apatite was treated at 100°C under normal atmospheric pressure for 3 h. After treatment, the apatite turned to needle-like nanocrystals (Mo et al., 2006). After washing to neutrality, the slurry containing 10 wt% nano-hydroxyapatite and 90% deionized water was chosen to make the composite membrane with chitosan (400 kDa; deacetylation 0.95). The tensile strength δ_o [MPa] and elongation rate ε_o [%] of wet membranes with hydroxyapatite to chitosan ratio of 4:6, at 30°C, 60°C, and 90°C were, respectively, 9.43 ± 0.39 and 56 ± 8.54; 10.56 ± 1.15 and 65 ± 11.09; and 4.96 ± 2.12 and 20 ± 7.07. The surface roughness and micropores of the composite membranes increase with the rise of nano-hydroxyapatite content, suitable for adhesion and growth of cells. Thanks to its nano size, the hydroxyapatite was distributed uniformly in the composite membranes. Chemical bonds were present between Ca ions and –OH groups of both hydroxyapatite and chitosan. Cell culture and MTT assays showed that the hydroxyapatite content influenced the proliferation of cells. The composite membranes had no negative effect on the bone marrow stromal cell morphology, viability, and proliferation, while it had good biocompatibility (Cheng et al. 2009).

The bone-regenerating efficacy of a nano-hydroxyapatite + collagen + poly(lactic acid) composite reinforced with chitosan fibers (83.8% deacetylated, 12.5 μm diameter, 550 MPa tensile strength) was evaluated in a goat shank model. Forty adult male goats with 40 mm defects in shank at the same anatomic site were divided into four groups. Besides the control group, three groups were implanted with porous poly(lactic acid), composite, and reinforced composite, respectively. Composite implants, with and without chitin fibers, were more effective in repairing the defects than poly(lactic acid) alone. However, only the chitosan-reinforced implants provided perfect recovery (in 15 weeks) with appropriate strength and high mineral density (Li et al. 2006).

Because the chitosan–polygalacturonan complexes are well known (Muzzarelli et al. 2004), the pectin polygalacturonan was expected to provide stronger interfacial interactions and improve the

mechanical properties of the composite. Thus nanocomposites of chitosan + hydroxyapatite + polygalacturonan have been synthesized via a biomimetic approach. Atomic force imaging of fractured and polished surfaces of the nanocomposite displayed chitosan-rich and polygalacturonan-rich domains made of smaller globular-shaped particles in which hydroxyapatite nanoparticles were embedded in the biopolymer matrix. The average size of the hydroxyapatite particles was found to be 34 nm; the elastic modulus of the composite was 23.62 GPa. Macro-mechanical tests showed a significant enhancement of elastic modulus, strain to failure, and compressive strength of the said composite over those containing either chitosan–hydroxyapatite or galacturonan–hydroxyapatite only (Verma et al. 2008).

It was possible to combine chitosan + 2-glycerophosphate salt formulations with bioactive glass nanoparticles in order to conceive novel injectable thermo-responsive hydrogels for orthopaedic, reconstructive, and regenerative medicine applications. The initial rheological properties and the gelation points of the developed organic–inorganic in situ thermosetting systems were revealed to be adequate for intracorporal injection. In vitro bioactivity tests, using incubation protocols in simulated body fluid, allowed the observation of bone-like apatite formation in the hydrogel formulations containing nanoparticles. The density of the apatite formed increased with increasing bioactive glass content and soaking time. These results indicate that the stimuli-responsive hydrogels could be used as temporary injectable scaffolds in bone tissue engineering (Couto et al. 2009).

Mesenchymal stem cells harvested from rabbits were induced into osteoblasts in vitro and were cultured for a week, statically loaded onto the porous hydroxyapatite + chitin matrices and implanted into bone defects of the rabbit femur for 2 months. The histology of explants showed bone regeneration with the biodegradation of the hydroxyapatite + chitin matrix. Similarly, green fluorescence protein transfected mesenchymal stem cells–induced osteoblasts were also loaded onto porous hydroxyapatite + chitin matrices and implanted into the rabbit femur. Mesenchymal stem cells–induced osteoblasts did not only proliferate but also recruited surrounding tissue to grow in. The hydroxyapatite + chitin matrix qualified for tissue-engineered bone substitutes (Ge et al. 2004).

While the ability of chitosan to bind growth factors and release them in a controlled fashion is well demonstrated in the above cited articles, the cationicity of chitosan can be further enhanced by a covalently linked imidazole group. The biochemical significance of imidazole addition is that this group inhibits thromboxane synthetase, acts as antioxidant, and facilitates intracellular buffering for the tissue healing process. This imidazole-linked chitosan material promoted mineralization, induced bone formation and filled critical size bone defects with the apposition of trabecular bone (Muzzarelli et al. 1994). Chitosan has been used to modify the surface properties of prosthetic materials for enhancing the attachment of osteoblasts. Titanium coated with chitosan via silane–glutaraldehyde exhibited increased osteoblast attachment and proliferation. The bond strength of chitosan coating with Ti was in the range of 1.5–1.8 MPa and its full degradation took 8 weeks, thus supporting the hypothesis that chitosan promoted osseo-integration of Ti devices commonly used for orthopedic implants, although chitosan bond strength was found to be less compared to calcium phosphate coatings (Bumgardner et al. 2003a,b).

The latter team developed a chitosan + nanocrystalline calcium phosphate composite scaffold and compared it to a plain chitosan scaffold to be used as a bone graft substitute. Composite and chitosan scaffolds were prepared by fusing microspheres of 500–900 μm in diameter: both kinds of scaffolds had porosities of 33%–35% and pore sizes between 100 and 800 μm. However, composite scaffolds were rougher and, as a result, had 20-fold larger specific surface area than chitosan scaffolds. The compressive modulus of hydrated composite scaffolds was significantly higher than chitosan scaffolds (9.29 ± 0.8 versus 3.26 ± 2.5 MPa), and composite scaffolds were tougher and more flexible than what has been reported for other chitosan + calcium phosphate composites or calcium phosphate scaffolds alone. Scaffolds contained partially crystalline hydroxyapatite with crystallinity 16.7% ± 6.8%, and crystallite size 128 ± 55 nm. On composite scaffolds, fibronectin adsorption was increased, and cell attachment was higher after 30 min, although attachment rates

were similar after 1 h. Osteoblast proliferation increased after 1 week of culture. These studies have demonstrated that composite scaffolds have mechanical properties and porosity sufficient to support ingrowth of new bone tissue, and cell attachment and proliferation data indicated that composite scaffolds are suitable for bone regeneration (Chesnutt et al. 2009).

17.5 GUIDED BONE REGENERATION

Guided bone regeneration is such a treatment that reconstructs new tissue by using a barrier membrane to guard the defected area from the invasion of other tissues, especially fibrous connective tissues. Being a relatively simple treatment, it received special attention and it was proposed for periodontal therapy where membranes prevent the apical migration of gingival epithelial cells into the bony defect site and promote the growth of progenitor bone and periodontal ligament cells (Nyman et al. 1982, Kostopoulos et al. 1994, Piattelli et al. 1996, Dupoirieux et al. 2001). Membranes for this purpose include Gore-Tex®, collagen membrane; Bio-Guide®, Vicryl Periodontal Mesh, polylactic acid sheet; and Guidor. General requirements for the barrier membranes with excellent biocompatibility in the GBR process are suitable mechanical strength, mechanical stability, optimal porosity, and biodegradability. A porous structure both at the surface and in the sublayer of the membranes is essential for cellular adaptation and sufficient nutrient permeation. Nonbiodegradable synthetic membranes, such as Gore-Tex®, required a secondary surgical procedure for retrieval, and this remains a significant drawback. In order to avoid the second-stage operation of removing the nonabsorbable membrane and to guarantee the continuous healing of tissue, the membrane should be able to be completely bioabsorbable after it has performed its function. In fact, biodegradable membranes, such as synthetic polyesters and collagen, do not require secondary surgery for membrane removal. However, the degradation products of the synthetic polymers reduce the local pH, accelerate the polymer degradation rate, and induce an inflammatory response. On the other hand, collagen is potentially immunogenic and can be expensive, and there can be great variations between the collagen batches (Vert et al. 1994, Lee et al. 2001).

Of course, suitable mechanical properties are essential for satisfactory applications and should accompany biodegradability: plain chitosan films failed in this respect because of poor mechanical resistance at the time of application. The preparation of chitosan-based composite films has to confer improvements of the mechanical properties: this was the scope of the studies on chitosan + silicate hybrid membranes mentioned below.

17.6 CHITOSAN WITH SILICATE COMPOSITES

Chitosan powder (medium molecular weight and degree of deacetylation 0.85), and tetramethylorthosilane were used to manufacture chitosan + silica xerogel hybrid membranes in which silica was dispersed at the nanoscale. The hybrid membranes showed superior mechanical properties to chitosan in the wet state and the rapid induction of calcium phosphate minerals in simulated body fluid, reflecting its excellent in vitro bone bioactivity. Osteoblasts adhered well and grow actively on the hybrid membrane to a level higher than that observed on the plain chitosan membrane. The alkaline phosphatase activity of the cells was also much higher on the hybrid than on the chitosan membrane. The in vivo bone regeneration around the membranes was assessed using a rat calvarial defect. After Masson–Trichrome staining, a recently formed bone area appeared in blue, whereas the calcified bones and remaining materials were stained in red. No inflammatory reaction was observed around either the chitosan or hybrid membranes. When the plain chitosan membrane was used to cover the calvarial defect, the latter was only partially healed with new bone, and a large portion of the membrane still remained. In contrast, when the hybrid membrane was used, the defect area was filled with thick new bone, and the membrane was almost entirely resorbed. For the histomorphometric analysis, the defect sites were observed at high magnification: the plain chitosan membrane was partially degraded and the remaining part was surrounded by collagen fibers,

bone marrow, and osteocyte cells. On the other hand, the hybrid membrane was almost completely degraded, and the defect was replaced by collagen fibers and new bone. The histomorphometric analysis indicated a significantly different bone formation rate between the hybrid and chitosan membranes. Remarkably, the chitosan + silica xerogel hybrid membrane induced the generation of new bone so effectively that the defect was almost completely healed in 3 weeks, whereas there was only 57% defect closure in the chitosan membrane (Lee et al. 2009).

Chitosan + silicate hybrids were also synthesized using gamma-glycidoxypropyltrimethoxy silane whose epoxy group react with the amino groups of chitosan. The cross-linking density was around 80% regardless of the amount of silane. The hydrophilicity of the hybrids increased except when the content exceeded the molar ratio of 1.5. The values of the mechanical parameters indicated that significant stiffening of the hybrids was obtained upon the addition of the silane while full flexibility was retained; the Young's modulus increased with higher quantities of the silane. The adhesion and proliferation of the MG63 osteoblast cells cultured on the chitosan hybrid surface were improved compared to those on the plain chitosan membrane, regardless of the silane concentration. Moreover, human bone marrow osteoblast cells proliferated on the chitosan hybrid surface and formed a fibrillar extracellular matrix. The same hydrogel derived from chitosan and said silane was characterized for the purpose of developing an injectable system for the application of Bonelike® using a resorbable vehicle usable in minimal invasive surgery. The Bonelike® graft is a bone substitute that mimics the inorganic composition of bone; this biomaterial was developed and characterized over the last decade. The mixture derived from chitosan and said silane existed in sol state at room temperature and formed a hydrogel at 37°C, whose degradation was controlled by the concentrations of both ingredients. The pH changes caused by the degradation of this hydrogel were small, so it did not cause any deleterious effect in vivo (Shirosaki et al. 2009a,b). When cultured with bone marrow cells, the hybrids showed abundant cell growth and matrix mineralization in the presence as well as in the absence of dexamethasone: this is a relevant observation because this compound is frequently added to cell cultures to improve the proliferation and/or differentiation of osteoblastic cells in a number of cell systems (Maniatopoulos et al. 1988).

Dexamethasone was impregnated in chitosan scaffolds using supercritical fluid technology, in order to improve the impregnation process, which in aqueous systems is jeopardized by the high viscosity of the chitosan solution and the low solubility of dexamethasone in water. Impregnation using supercritical fluid technology has proven to be feasible when the drug is soluble in carbon dioxide and the polymer can be swollen by the supercritical fluid; a pure product, free of residual solvents is obtained, because the only solvent present is the volatile carbon dioxide. Supercritical fluids, especially supercritical carbon dioxide, have prime roles in the development of clean processes for the preparation of drug-loaded polymeric supports. In this context, chitosan sponges were prepared from a 4% solution of chitosan in a 2% acetic acid Solution, contained into cylindrical moulds, which were frozen at −80°C and lyophilized. The scaffolds were neutralized with 0.1 M NaOH and washed with water, to be frozen and lyophilized again, and then they were impregnated with dexamethasone at a pressure of 8.0–14.0 MPa and a temperature of 35°C–55°C. The highest loading was achieved at 8.0 MPa and 35°C, thus supercritical fluid impregnation proved to be useful in this area (Duarte et al. 2009).

17.7 OSTEOGENIC DIFFERENTIATION AND BONE MORPHOGENETIC PROTEINS

Mesenchymal stem cells are a valuable therapeutic tool in tissue engineering because they proliferate and differentiate into distinct cellular phenotypes, such as osteoblasts, chondrocytes, adipocytes, and muscle cells. Mesenchymal stem cells have been initially identified in bone marrow as nonhematopoietic stem cells, called bone marrow-derived stem cells. Their osteogenic differentiation potential was described by Maniatopoulos et al. (1988). The adipose tissue, often removed during

plastic surgery, might become an alternative source of mesenchymal stem cells, mainly because adipose-derived stem cells can be extracted from adipose tissue isolates in large quantities, potentially eliminating the need for in vitro expansion. Reports on their use are controversial, however.

The development of novel strategies that stimulate stem cells to become osteoblasts in vitro and in vivo, and that provide a more effective treatment route with diminished complications seems to attract attention for further exploitation. In this context, nanocarriers that possess high cellular uptake efficiency to deliver and target drugs may deserve investigations, since they can allow the modulation of the cellular functions in an effective manner *ex vivo*, and maintain the cellular phenotype in vivo upon reimplantation. The effect of dexamethasone-loaded carboxymethyl chitosan + poly(amidoamine) dendrimer nanoparticles on the proliferation and osteogenic differentiation of rat bone marrow stromal cells in vitro has been studied with the aid of O-carboxymethyl chitosan prepared according to Chen and Park (2003). The stromal cells seeded onto the surface of hydroxyapatitte scaffolds differentiated into osteoblasts when cultured in the presence of dexamethasone-loaded nanoparticles, and enhanced osteogenesis by increasing the alkaline phosphatase activity and the mineralization of the extracellular matrix. The pre-incubation of stem cells with these kinds of nanoparticles allowed the delivery of dexamethasone inside the cells and influenced their fate (Oliveira et al. 2009b).

In the context of current studies dealing with the roles of stem/progenitor cells in osteogenesis, chitosan promoted osteogenic progenitor cell recruitment and attachment thus facilitating bone formation (Kim et al. 2002). When cultured mesenchymal stem cells were treated in vitro with chitosan, the treated cells showed significantly higher averages of colonies per well than that of the control, suggesting that chitosan may promote the differentiation of osteoprogenitor cells and bone formation. The calcium phosphate/chitosan coating also showed an improved bone marrow stromal cell attachment. The chitosan–alginate gel + mesenchymal stem cells + bone morphogenetic protein-2 composites were found to stimulate new bone formation when injected into the mouse (Park et al. 2005).

While the applications and delivery strategies for bone morphogenetic proteins were reviewed by Kirker-Head (2000) no mention of chitosan can be found in that article. Today, however, a relatively large number of experimental works, besides those already mentioned above, make use of a variety of chitosans to deliver human and recombinant bone morphogenetic proteins that induce the differentiation of multipotential mesenchymal cells, pluripotent murine stem cell cultures, and rat bone marrow stromal cell as well as the proliferation and maturation of osteoblast populations. The quantity and quality of newly formed bone, stimulated by recombinant human BMP-2 in combination with either mono-olein or chitosan acetate gel as carriers, were evaluated in defects made in 36 Wistar rat mandibles. There were statistical differences between groups of animals receiving or not the rhBMP-2. Both mono-olein and chitosan gels were adequate carriers for defect filling and control of protein release (Issa et al. 2008). Although rhBMP-2 seems to induce the formation of new bone tissue by itself, association to an immobilizing carrier during a time sufficient to elicit a cellular response has a potentiating effect: the higher osteoinductive capacity of mono-olein or chitosan gel associated to rhBMP-2, confirms observations by other investigators who considered that there is a direct relationship between the osteoinductive action of the morphogenetic protein and the carrier retention capacity. Many studies have shown that bone repair is optimized by association of the protein to a sustained release carrier. Sustained release dosages of rhBMP-2 from 0.5 to 115 mg were tested to find an optimal value around to 20 mg, not only in terms of efficacy but also cost (Abarrategi et al. 2008). Coated calcium sulfate pellets combined with rhBMP-2 facilitated new bone formation in vivo (Lee et al. 2002, Cui et al. 2008).

Bone morphogenetic protein-2 has been widely used as an effective growth factor in bone tissue engineering. However, large amounts of BMP-2 are required to induce new bone and the resulting side effects limit its clinical application. Sulfated polysaccharides, such as heparin and heparan sulfate have been found to modulate BMP-2 bioactivity and play pivotal roles in bone metabolism. Several sulfated chitosans were synthesized by regioselective reactions firstly. Using C2C12

myoblast cells as in vitro models, the enhanced bioactivity of BMP-2 was attributed primarily to the stimulation by 6-O-sulfated chitosan, while 2-N-sulfate group had less activation. A low dose of 2-N,6-O-sulfated chitosan (26SCS) showed significant enhancement on the alkaline phosphatase activity and the mineralization formation induced by BMP-2, as well as the expression of ALP and osteocalcin mRNA. Dose-dependent effects on BMP-2 bioactivity were observed in both sulfated chitosan and heparin. Compared with native heparin, 26SCS showed much stronger simultaneous effects on the BMP-2 bioactivity at low dose. The BMP-2 ligand bound to its receptor was enhanced by low dose of 26SCS, whereas weakened by the increasing amounts of 26SCS. Furthermore, the simultaneous administration of BMP-2 and 26SCS in vivo induced larger amounts of ectopic bone formation in a dose-dependent manner compared with BMP-2 alone. These findings clearly indicate that 26SCS is a more potent enhancer for BMP-2 bioactivity to induce osteoblastic differentiation in vitro and in vivo by promoting BMP-2 signaling pathway (Zhou et al. 2009).

To develop a novel tissue engineering scaffold with the capability of releasing BMP-2-derived synthetic peptide, porous poly(lactic acid) + chitosan microspheres composites containing different quantities of chitosan microspheres were prepared by a thermally induced phase separation method. FTIR analysis revealed that there were strong hydrogen bond interactions between the PLA and chitosan components. The introduction of less than 30% chitosan microspheres (on PLA weight basis) did not remarkably affect the morphology and porosity of the poly(lactic acid) + chitosan microspheres scaffolds. The compressive strength of the composite scaffolds increased from 0.48 to 0.66 MPa, while the compressive modulus increased from 7.29 to 8.23 MPa as the microsphere contents increased to 50%. The dissolution of chitosan was preferential than PLA matrix and the inclusion of chitosan microspheres could neutralize the acidity of poly(lactic acid) degradation products. The synthetic peptide was released from the new scaffolds in a temporally controlled manner, depending on the degradation of PLA (Niu et al. 2009).

Chondrocyte cultures in the integrated gelatin + chitosan + hyaluronan crosslinked scaffold integrated with 50% poly(lactide-co-glycolide) microspheres demonstrated that the cells could proliferate and secrete extracellular matrix at the same level as in the control scaffold (Tan et al. 2009). As a step forward, the recombinant human bone morphogenetic protein-2 was encapsulated in poly(lactide-co-glycolide) biodegradable microspheres, which were then dispersed in a chitosan + collagen composite scaffold. The effect of rhBMP-2 encapsulated scaffolds on enhancing bone formation through implantation in dogs' mandibles was defined upon the histological examination of the regenerated bone after 4 weeks of implantation. Due to PLGA microspheres, the said scaffold exhibited lower values of porosity and swelling rate, but higher release than control. Bone density, bone-implant contact, and bone-fill values in vivo demonstrated that the composite scaffold-induced bone regeneration more quickly and that it was promptly replaced by new bone. It was concluded that this sustained carrier scaffold based on microspheres was more effective to induce implant osseointegration (Shi et al. 2009).

The effects of a combined chitosan and collagen matrix on osteogenic differentiation of rat-bone-marrow stromal cells was investigated on four study groups: collagen, chitosan, 1:1 chitosan–collagen, and 1:2 chitosan–collagen sponges fabricated by freeze-drying. Bone-marrow stromal cells seeded on the sponges and cultivated in mineralized culture medium for 27 days attached to the sponges: the expression of alkaline phosphatase and osteocalcin (that reveal the progress of differentiation) in collagen and chitosan–collagen sponges was greater than that on chitosan sponges; on the other hand, chitosan and chitosan–collagen sponges showed higher resistance to enzymatic degradation than collagen sponges. The best chitosan–collagen ratio was 1:1 because it promoted the osteoblastic differentiation of bone-marrow stromal cells, and improved the mechanical and physical properties of the sponges (Arpornmaeklong et al. 2008).

Mouse osteoblasts and fibroblasts were grown on chitosan in the presence of serum. Cell attachment and immunofluorescence analysis were done to analyze phenotypic profiles. Osteoblast attachment at 1 h was significantly greater than that with fibroblasts. At 24 h, levels of cell attachment for fibroblasts increased and became similar to those in osteoblast cultures at 1 and 24 h. Fibroblasts showed

a heterogeneous population of round and semi-spread cells, but in comparison, osteoblasts displayed phenotypes that were well spread with a developed cytoskeleton (Fakhry et al. 2004).

For an osteochondral defect, one should consider the need for simultaneous regeneration of both cartilage and subchondral bone. Several strategies for developing hybrid constructs for osteochondral tissue engineering are (1) cell culturing performed independently on two sides, which are integrated before implantation; (2) two different cell sources seeded in the two sides of a single- or double-phase scaffold, and cultivated in a special bioreactor with two separated chambers; and ideally (3) common progenitor cells seeded in the two sides of a biphasic scaffold that contains different differentiation agents and then cultivated in a bioreactor with one or two chambers (Schaefer et al. 2000, 2002, Wendt et al. 2005, Huang et al. 2007, Mano and Reis 2007, Martin et al. 2007).

Double-phase scaffolds based on hydroxyapatite dispersed into chitosan crosslinked with glutaraldehyde were developed based on the optimization of both polymeric and composite scaffolds: tomography was carried out to accurately quantify porosity, interconnectivity, ceramic content, particle, and pore size. The scaffolds were highly interconnected, presented the ideal pore size range to be morphometrically suitable for the proposed applications, and were mechanically stable in the wet state even under dynamic compression. The obtained elastic modulus was 4.21 ± 1.04, 7.98 ± 1.77, and 6.26 ± 1.04 MPa for polymeric, composite, and bilayered scaffolds, respectively. Bioactivity studies using a simulated body fluid and a simulated synovial fluid were conducted in order to assure that the polymeric component for chondrogenic part would not mineralize, as confirmed by scanning electron microscopy, inductively coupled plasma-optical emission spectroscopy and energy-dispersive spectroscopy for different immersion periods. The assays were carried out also under dynamic conditions using, for this purpose, a specifically designed double-chamber bioreactor, aiming at a future osteochondral application. It was concluded that chitosan-based bilayered scaffolds produced by particle aggregation overcome any risk of delamination of both polymeric and composite parts designed for mechanically stable chondrogenic and osteogenic components, respectively (Malafaya et al. 2009).

Other authors are in favor of the application of a single-phase material, endowed with gradients of molecular, structural, and functional properties. For example, Vunjak-Novakovic et al. (2005) proposed a single-phase silk-based scaffold functionalized by covalently binding growth factors with opposing gradients of a chondrogenic factor (IGF-I) and an osteogenic factor (BMP-2) for tissue engineering of osteochondral grafts. The opposite gradients of these two different growth factors in the same scaffold are proposed in order to mimic the most suitable concentration of IGF-I for cartilage at one end and the most suitable concentration of BMP-2 for bone at the other end.

Circulating progenitor cells are known to home to various organs to repair injured tissues or to routinely replace old cells and maintain tissue integrity. Similarly, circulating progenitor bone cells can home to a bone implant, differentiate, and eventually osteointegrate with the prosthesis, thus helping reduce the risk of implant failure. Immobilized bone morphogenetic protein-2 on chitosan-grafted titanium support enhanced bone marrow–derived mesenchymal stem cell adhesion onto the substrate surface and further induced their differentiation into osteoblasts. The chitosan-Ti support is able to release slowly the adsorbed BMP2. Based on the fact that alizarin red staining revealed the presence of calcium deposits in the differentiated cells, the Ti–chitosan–BMP2 supports exerted an osteoconductive effect (Lim et al. 2009).

17.8 CONCLUSION

In the short lapse of a few years, the research works on bone regeneration with the aid of bone cements have become more refined in terms of the effects of novel chitosan scaffolds on the cells involved in the healing process. The use of nano-hydroxyapatite as well as other inorganics in conjunction with variously modified chitosans is greatly contributing to the advancement of chitosan composites for bone healing.

The cartilage regenerative medicine has also evolved during the last decade. While the early technology (autologous chondrocyte transplantation) involved the transplantation of in vitro expanded chondrocytes to cartilage defects, the new technology involves the seeding of chondrocytes in a scaffold and offers several advantages such as arthroscopic implantation, in vitro preliminary differentiation of cells, and implant stability. Bioreactors enhance the biochemical and mechanical properties of the engineered tissues with adequate mass transfer and physical stimuli, as reviewed by Concaro et al. (2009). The development of automatic culture systems and noninvasive monitoring of matrix production are expected to take place during the next few years in order to improve the cost effectivity of the products. Although biological repair of the degenerate intervertebral disc would be the ideal treatment, there is a lack of a generally accepted scaffold for tissue engineering of intervertebral discs and little is known on how to differentiate mesenchymal stem cells to a disc-like phenotype, but Mwale et al. (2009) showed that 2.5% medical-grade chitosan cross-linked with genipin (Muzzarelli, 2009c) might be a promising scaffold for this purpose.

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18 Antioxidative Activity of Chitosan, Chitooligosaccharides and Their Derivatives

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CONTENTS

18.1 INTRODUCTION

Chitin is a naturally abundant mucopolysaccharide distributed in the shell of crustaceans, in the cuticle of insects, and also in the cell wall of some fungi and microorganisms. Chitin consists of 2-acetamido-2-deoxy-(1-4)-β-D-glucopyranose residues (*N*-acetyl-D-glucosamine units) that has intra- and intermolecular hydrogen bonds and is a water-insoluble material resembling cellulose in its solubility. It may be regarded as cellulose with hydroxyl group at position C-2 replaced by an acetamido group. In addition, it has a similar function to cellulose in plants, serving as a supportive and protective material. Chitin is a polysaccharide that is a white, hard, inelastic nitrogenous compound, and the major source of surface pollution in the coastal areas. Chitin production is estimated approximately 1×10^9 metric tons annually and it is the second most abundant natural biopolymer.

Chitosan is an *N*-deacetylated derivative of chitin and consists of 2-amino-2-deoxy-(1-4)-β-D-glucopyranose residues (D-glucosamine units), and water-soluble by making salt with various acids on the amino group of D-glucosamine unit. Additionally, partially acetylated chitosan having about 50% D-glucosamine units is only able to dissolve in water (Aiba 1989). Chitin and chitosan are of commercial interest due to their high percentage of nitrogen (6.89%) compared with synthetically substituted cellulose (1.25%).

Until the mid-1980s, major applications of chitosan were centered on sludge dewatering, food processing, and metal ion chelation. Recently, new applications, however, are concentrated on producing high-value products like cosmetics, drug carriers, food additives, semipermeable membranes, and pharmaceuticals. They have been developed as new physiological bioactive materials since they possess various biological activities such as antibacterial activity (Allan and Hadwiger 1979, Hadwiger and Beckman 1980, Walker-Simmons et al. 1983, Hirano and Nagao 1989, Jeon and Kim 2000, Kim et al. 2000, Jeon et al. 2001, Park et al. 2004a,b), hypocholesterolemic activity (Sugano et al. 1980, 1988, Hirano et al. 1990, Maezaki et al. 1993), and antihypertensive activity (Okuda et al. 1997, Park et al. 2003, 2008). In recent studies, there has been more of interest for converting chitin and chitosan

to their oligomers or oligosaccharides. Even though chitin and chitosan are known to have very interesting functional features, direct absorption in the human intestine is doubtful because of their physicochemical properties. Most animal intestines including human gastrointestinal tract do not possess enzymes which digest the β-glucosidic linkage in chitin and chitosan, therefore they are excreted unchanged in the feces without any degradation or significant absorption.

Chitosan oligosaccharides (COSs) have lower viscosity with relatively smaller molecular size that makes them water-soluble and readily absorbable in the *in vivo* systems. They also possess additional functional properties like antitumor activity (Suzuki et al. 1986, Tsukada et al. 1990, Suzuki 1996, Jeon and Kim 2002), immuno-stimulating effects (Tokoro et al. 1988, Jeon and Kim, 2001), enhancing protective effects against infection with some pathogens associated in mice (Tokoro et al. 1988), antifungal activity (Hirano and Nagao 1989), and antimicrobial activity (Park et al. 2004a,b).

In this chapter, antioxidative activity of chitosan and their derivatives containing COSs has been reviewed.

18.2 OVERVIEW ON ANTIOXIDANTS

The term antioxidant is defined as any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate. Antioxidant can act at different levels in an oxidative sequence. This may be illustrated by considering one of the many mechanisms, by which oxidative stress can cause damage by stimulating the free radical chain reaction of lipid peroxidation. Free radical chain reactions within a material may be inhibited either by adding chemicals that retard the formation of free radicals (preventive antioxidants) or by introducing substances that compete for the existing radicals and remove them from the reaction medium (chain-breaking antioxidants).

Lipid oxidation is of great concern to the food industry and consumers because it leads to the development of undesirable off-flavors and potentially toxic reaction products (Maillard et al. 1996). Synthetic antioxidants such as butylated hydroxyanisol, butylated hydroxytoluene, tertiary-butylhydroquinone, and propyl gallate may be added to food products to retard lipid oxidation (Wanita and Lorenz 1996). However, use of synthetic antioxidants in food products is under strict regulation due to the potential health hazards caused by such compounds (Hettiarachchy et al. 1996). Therefore, search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers. Especially, free radical-mediated lipid peroxidation, oxidative stress, and antioxidants have been the focus of popular research in recent history. Under normal conditions, reactive oxygen species (ROS) and free radicals are effectively eliminated by antioxidant defense systems such as antioxidant enzymes and nonenzymatic factors. However, under pathological conditions, the balance between generation and elimination of ROS is disrupted. As a result, biomacromolecules including DNA, membrane lipids, and proteins are damaged by ROS-mediated oxidative stress. Uncontrolled generation of free radicals that attack membrane lipids, protein, and DNA, is believed to be linked with health disorders such as diabetes mellitus, cancer, neurodegenerative diseases, gastric ulcers, ischemic reperfusion, arthritis, and inflammatory diseases (Debashis et al. 1997). Scavengers of free radicals and ROS are substances that delay or prevent the oxidation of cellular oxidizable substrates. This mechanism attacking free radicals and ROS is triggered by the prevention of free radical and ROS generation or by the activation of a battery of detoxifying proteins.

18.3 ANTIOXIDATIVE ACTIVITY OF CHITOSAN, CHITOOLIGOSACCHARIDES, AND THEIR DERIVATIVES

Chitosan has attracted attention as a biomedical and food material, owing to its unique biological benefits such as antitumor, antimicrobial, and antioxidative and immuno-enhancing properties. In addition, their various activities are affected by molecular weight (MW) or chain length and degree of deacetylation (DD) with their low toxicity.

18.3.1 Chitosan

Living organisms are constantly exposed to reactive oxygen species (ROS) formed as by-products of normal respiration, metabolism and autoxidation of xenobiotics, or resulting from stress imposed by a range of diseases (Halliwell and Gutteridge 2007). The formation of ROS is essential to maintain cell homeostasis and living organisms achieve this by a system of antioxidant defense, maintaining a balance between oxidative challenge and protection. Excessive production of ROS results in oxidative stress, leading to activation of specific signaling pathways and general damage. Chitosan is able to function as an effective antioxidant. Park et al. (2004a,b) prepared three kinds of partially deacetylated hetero-chitosans such as 90% deacetylated chitosan, 75% deacetylated chitosan, and 50% deacetylated chitosan from crab chitin, and measured their antioxidative activity using electron spin resonance (ESR) spectromer. The ESR spectrum of alkyl, hydroxyl, and superoxide radical is shown in Figure 18.1. All the hetero-chitosans examined were found to possess alkyl, hydroxyl, and superoxide-radical scavenging activity, and 90% chitosan with relatively high degree of deacetylation showed the highest radical scavenging effects on the hydroxyl and superoxide radicals. In addition, the effect of these hetero-chitosans depends on their degree of deacetylation and concentration. Yen et al. (2008) reported that various crab chitosan were prepared by alkaline *N*-deacetylation of crab chitin for 60 (C60), 90 (C90), and 120 (C120) min and antioxidative activity of the prepared chitosans exhibited antioxidative effects of 58.3%–70.2% at 1.0 mg/mL concentration. The C120 with more amino groups on C-2 position showed the highest antioxidative activity (Table 18.1). Generally, the degree of deacetylation was correlated with *N*-acetylation times. These results suggest that hetero-chitosans eliminate various free radicals by the action of nitrogen on the C-2 position of chitosan.

18.3.2 Chitooligosaccharides

Although chitosan is known to have various biological properties, it is poorly soluble in acidic medium of above pH 6.5. Therefore, many studies on chitosan have attracted interest for converting it to chitooligosaccharides (COSs). The COSs have lower viscosity and relatively small molecular size that makes them water-soluble and readily absorbable in the *in vivo* systems. They also possess

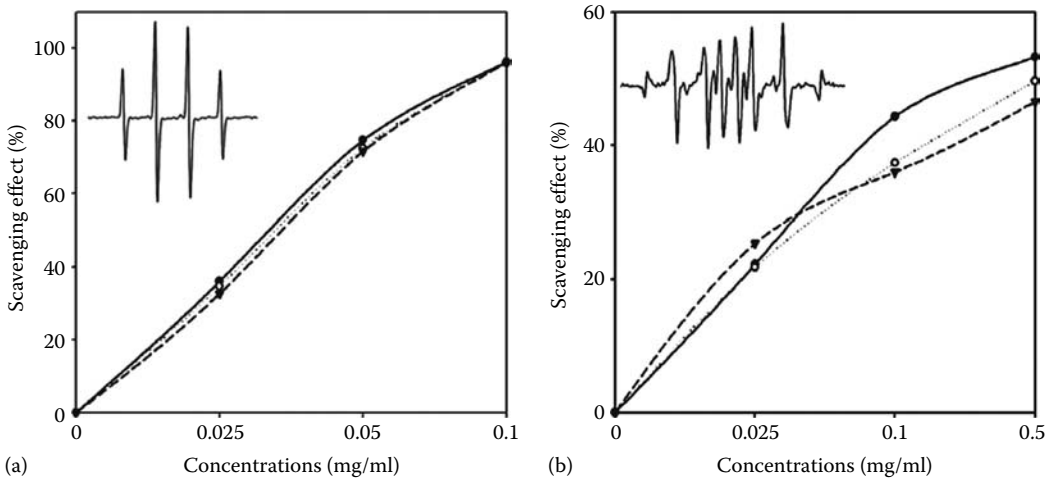

FIGURE 18.1 Scavenging effects of 90% chitosan(●), 75% chitosan (○), and 50% chitosan (▾) on hydroxyl radical obtained in Fenton reaction system (a) and on superoxide radical obtained in hypoxanthine–xanthine oxidase system (b) at various concentrations of hetero-chitosans. Inset shows ESR spectrum of the spin adduct of DMPO-OH (a) and superoxide radical (b). (From Park, P.J. et al., *Carbohydr. Polym*., 55, 17, 2004c. With permission.)

TABLE 18.1
EC_{50} Values of Chitosans from Crab Shells in Antioxidant Properties

	EC50[a] (mg Extract/mL)				
	C60	**C90**	**C120**	**CS**	**CC**
Antioxidant activity	1.32 ± 0.01 A[b]	0.72 ± 0.02 C	0.52 ± 0.01 D	0.77 ± 0.01 B	0.72 ± 0.01 C
Reducing power	20.00 ± 0.03 A[c]	13.46 ± 0.02 E[c]	13.75 ± 0.01 D[c]	16.25 ± 0.02 C[c]	16.43 ± 0.01 B[c]
Scavenging ability on DPPH radicals	16.30 ± 0.08 A[c]	11.67 ± 0.02 B[c]	9.13 ± 0.02 E	10.75 ± 0.02 C[c]	10.22 ± 0.01 D[c]
Scavenging ability on OH radicals	0.08 ± <0.01 A	0.08 ± <0.01 A	0.07 ± <0.01 B	0.07 ± <0.01 B	0.06 ± <0.01 C
Chelating ability on ferrous ions	0.68 ± 0.02 A	0.62 ± 0.01 B	0.57 ± 0.01 D	0.61 ± 0.01 C	0.61 ± 0.01 C

Source: Yen, M.T. et al., *Carbohydr. Polym.*, 74, 840, 2008. With permission.

[a] EC_{50} value: the effective concentration at which the antioxidant activity was 50%; the absorbance was 0.5 for reducing power; 1,1-diphenyl-2-picrylhydrazyl (DPPH) or hydroxyl (OH) radicals were scavenging by 50%; and ferrous ions were chelated by 50%, respectively. EC_{50} value was obtained by interpolation from linear regression analysis.

[b] Each value is expressed as mean standard deviation ($n = 3$). Means with different letters within a row are significantly different ($P < .05$).

[c] Obtained by extrapolation from linear regression analysis.

additional functional properties like antitumor activity, immuno-stimulating effects, and antimicrobial activity (Jeon and Kim 2002, Park et al. 2004a,b). Je et al. (2004) have prepared nine kinds of hetero-COSs from partially different deacetylated chitosans (50%, 75%, and 90% chitosan) by two enzymatic reactions using chitosanase and cellulose in an ultrafiltration membrane reactor system with relatively higher molecular weights (molecular weights, 5000–1000 Da; 90-HMWCOS, 75-HMWCOS, and 50-HMWCOS), medium molecular weights (molecular weights, 1000–500 Da; 90-MMWCOS, 75-MMWCOS, and 50-MMWCOS), and lower molecular weights (molecular weights, below 100 Da; 90-LMWCOS, 75-LMWCOS, and 50-LMWCOS). Additionally, free radical scavenging activity of the hetero-COSs was investigated on DPPH, hydroxyl, alkyl, and superoxide radicals using an ESR spectromer. The ESR results revealed that 90-MMWCOS, which is having relatively medium molecular weight prepared from 90% deacetylated chitosan, showed the highest scavenging activity on all tested radicals. Also, the radical-scavenging activity of hetero-COSs was increased in a dose-dependent fashion, and it was dependent on their degree of deacetylation values and molecular weights (Figures 18.2 and 18.3). Kim and Thomas (2007) reported the antioxidative activity of chitosans with different molecular weights (30, 90, and 120 kDa) in salmon where the chitosan with lowest MW (30 kDa) exhibited highest antioxidative activity. The inhibition of protein oxidation by ROS would confer benefit to living organisms exposed to oxidative stress, because oxidized proteins are associated with many diseases and can propagate ROS-induced damage. Anraku et al. (2008) have reported that 2800 Da COSs was effective in preventing formation of carbonyl and hydroperoxide groups in human serum albumin exposed to peroxyl radicals (Figure 18.4).

Free radicals with major species of ROS are unstable, and react readily with other groups or substances in the body, resulting in cell damage and hence human disease. Therefore, removal of free radical and ROS is probably one of the most effective defenses of a living body against various diseases. Beneficial effects of antioxidants are well known in scavenging free radical and ROS before or preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation. It is generally considered that the

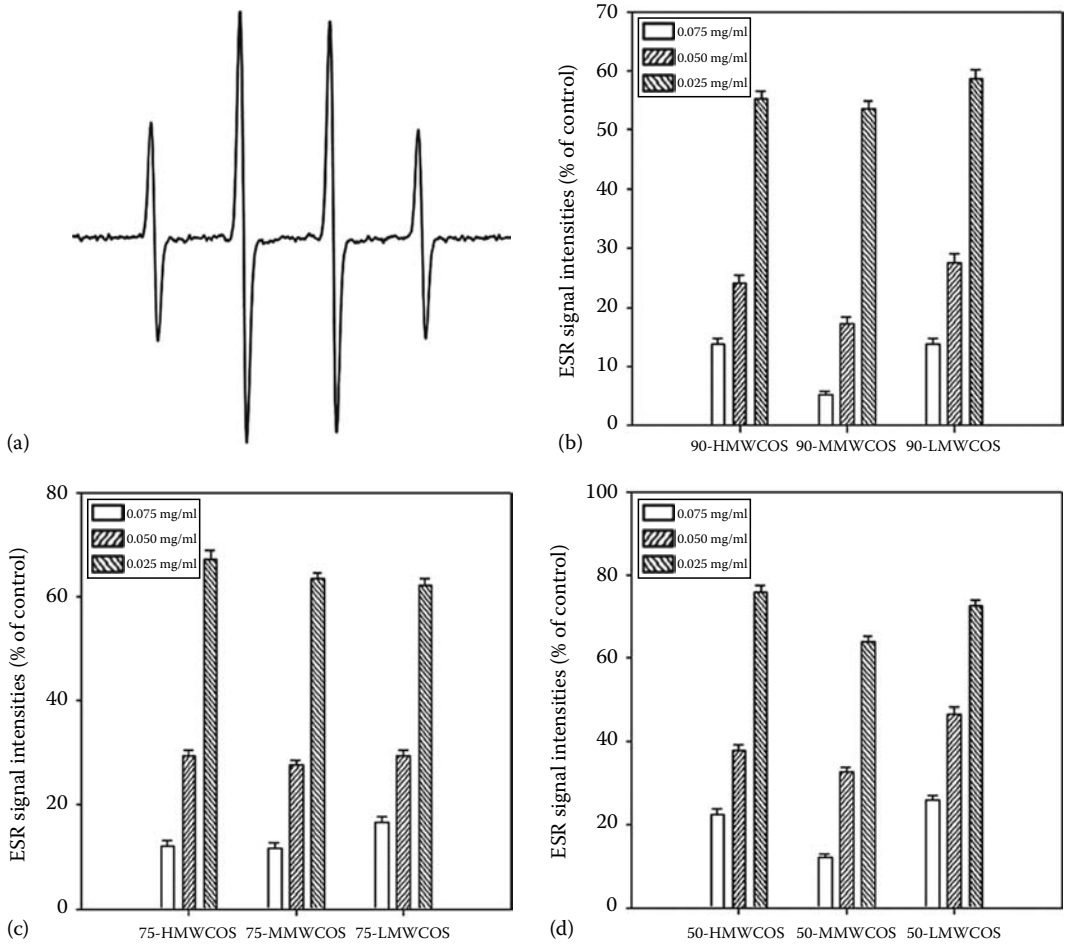

FIGURE 18.2 Hydroxyl-radical scavenging activity of hetero-chitooligosaccharides. (a) ESR spectrum of hydroxyl radical obtained in Fenton reaction system; (b) DPPH scavenging activity of 90-HMWCOS, 90-MMWCOS and 90-LMWCOS in various concentrations; (c) DPPH scavenging activity of 75-HMWCOS, 75-MMWCOS, and 75-LMWCOS in various concentrations; (d) DPPH scavenging activity of 50-HMWCOS, 50-MMWCOS and 50-LMWCOS in various concentrations. Values represent means ± SE (n = 3). (From Je, J.Y. et al., *Food Chem. Toxicol*., 42, 381, 2004. With permission.)

inhibition of lipid peroxidation by an antioxidant may be due to the free-radical scavenging activity. Lipids of biological membranes, especially those in the spinal cord and brain containing highly oxidizable polyunsaturated fatty acids, are particularly affected. Moreover, the brain contains considerable amounts of prooxidant transition metal ions and utilizes a lot of oxygen. These properties set the stage for the generation of free radical and ROS the subsequent acute cellular injury.

Therefore, the antioxidative activity of COSs was dependent on their degree of deacetylation values and molecular weights.

18.3.3 Chitosan and COSs Derivatives

Although chitosan has very strong biological activities in many fields, the water-insoluble property of chitosan is disadvantageous for its wide application. Therefore, chitosan derivatives with water-soluble and functional property have been developing for food additives and new drug candidates. As shown in Figure 18.5, amino-derivatized chitosan derivatives were prepared in addition of amino

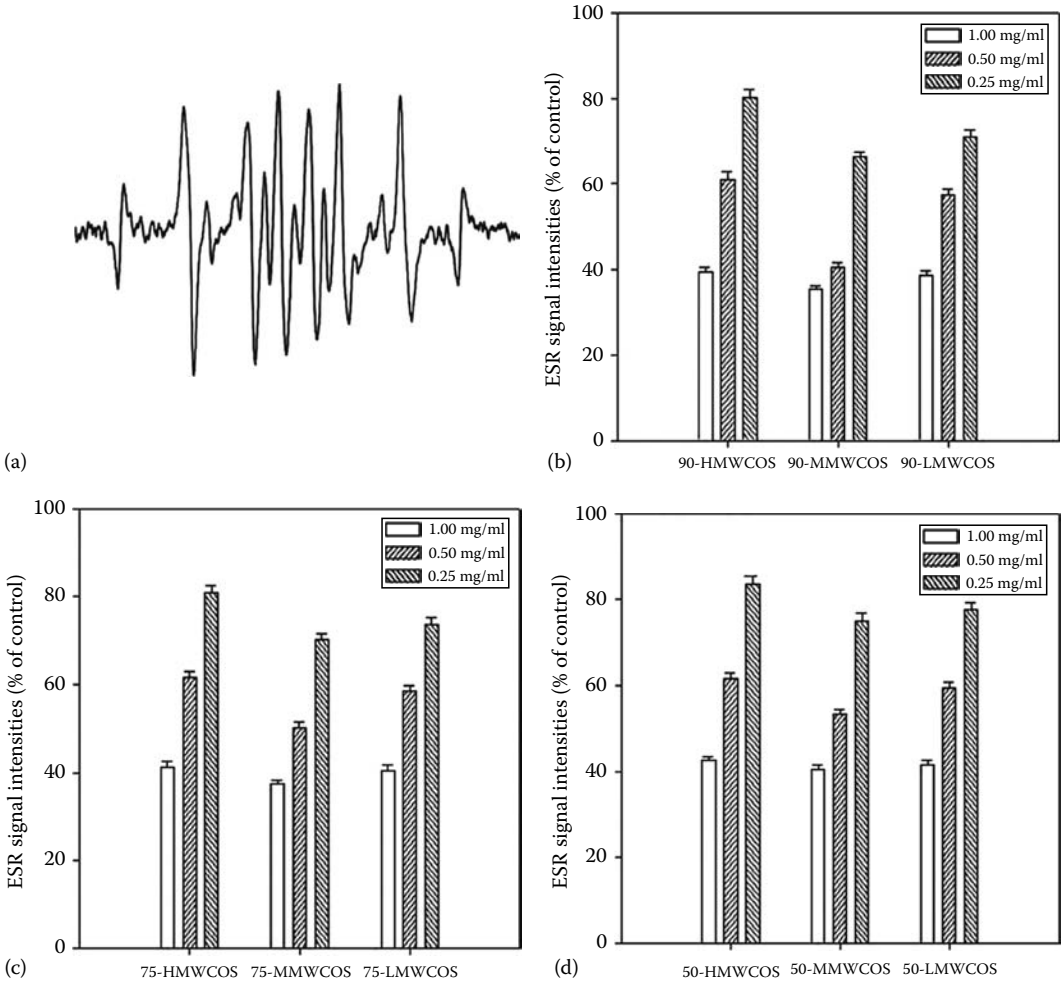

FIGURE 18.3 Superoxide-radical scavenging activity of hetero-chitooligosaccharides. (a) ESR spectrum of superoxide radical obtained in HPX-XOD system; (b) Superoxide-radical scavenging activity of 90-HMWCOS, 90-MMWCOS and 90-LMWCOS in various concentrations; (c) Superoxide-radical scavenging activity of 75-HMWCOS, 75-MMWCOS, and 75-LMWCOS in various concentrations; (d) Superoxide-radical scavenging activity of 50-HMWCOS, 50-MMWCOS and 50-LMWCOS in various concentrations. Values represent means ± SE (n = 3). (From Je, J.Y. et al., *Food Chem. Toxicol.,* 42, 381, 2004. With permission.)

biological groups at a hydroxyl site in the chitosan backbone from 50% and 90% deacetylated chitosan, and the free-radical scavenging activity on hydroxyl and superoxide radicals depended on their degree of deacetylation and substituted group (Je and Kim 2006).

Xie et al. (2001) have reported that the water-soluble chitosan derivatives prepared by graft copolymerization of maleic acid sodium onto hydroxypropyl chitosan and carboxymethyl chitosan sodium, exhibit scavenging activities against hydroxyl radicals.

Five kinds of Schiff bases of chitosan and carboxymethyl chitosan (CMTS) have been prepared, and their respective antioxidant effect was dependent on contents of active hydroxyl and amino groups in molecular chains (Guo et al. 2005).

Matsugo et al. (1998) reported that, three different water-soluble chitosan derivatives obtained by the acylation of chitosan inhibited thiobarbituric acid reactive substance formation in *t*-butylhydroperoxide and benzoyl peroxide induced lipid peroxidations.

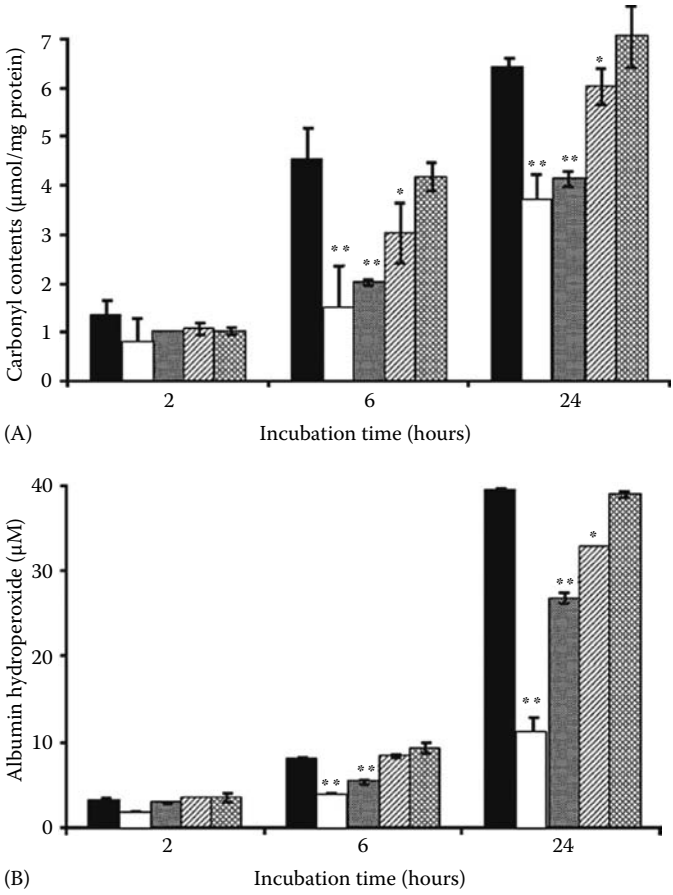

FIGURE 18.4 Inhibition of oxidation of human serum albumin by antioxidants. Solutions of 20 μM HSA were exposed at 37°C to 40 mM AAPH for various times and the degree of protein oxidation was measured by carbonyl (panel A) and hydroperoxide assays (panel B). The bars indicate levels of oxidized HSA formed after subtraction of control values. HSA only (■), Vit C (0.5 mg/mL) (□), LMWC (5 mg/mL) (▦), GlcN (5 mg/mL) (▨), and GlcNAc (5 mg/mL) (▩). *$P < .05$, **$P < .01$, compared to HAS in the absence of antioxidants. (From Anraku, M. et al., *Int. J. Biol. Macromol.*, 43, 159, 2008. With permission.)

Guo et al. (2006) have synthesized *N*-substituted chitosan and quaternized chitosan and evaluated their antioxidative activity on hydroxyl radicals. It has been found that the antioxidative activity of quaternized chitosan derivatives was higher than that of *N*-substituted chitosan derivatives.

In chitosan, the two kinds of hydroxyl groups at C-3 and C-6, and part of the relationship between the two kinds of hydroxyl groups in chitosan at C-3 and C-6 and their antioxidative activity has been studied previously by Guo et al. (2006). The sulfanilamide derivatives of chitosan or chitosan sulfates were prepared and the superoxide-radical scavenging activity was measured. The superoxide-radical scavenging activity of sulfanilamide derivatives of chitosan and chitosan sulfates was stronger than those of original chitosan and chitosan sulfates (Zhong et al. 2007). In addition, 2-(4(or 2)-hydroxyl-5-chloride-1, 3-benzene-di-sulfanimide)-chitosan was prepared, and their antioxidative effects were studied. Low molecular weight 2-(4(or 2)-hydroxyl-5-chloride-1, 3-benzene-di-sulfanimide)-chitosan had stronger scavenging activity on superoxide and hydroxyl radicals than those of high molecular ones (Zhong et al. 2008). The antioxidative activity of chitosan and its derivatives were affected by the charge properties of substituting groups and their degree of deacetylation.

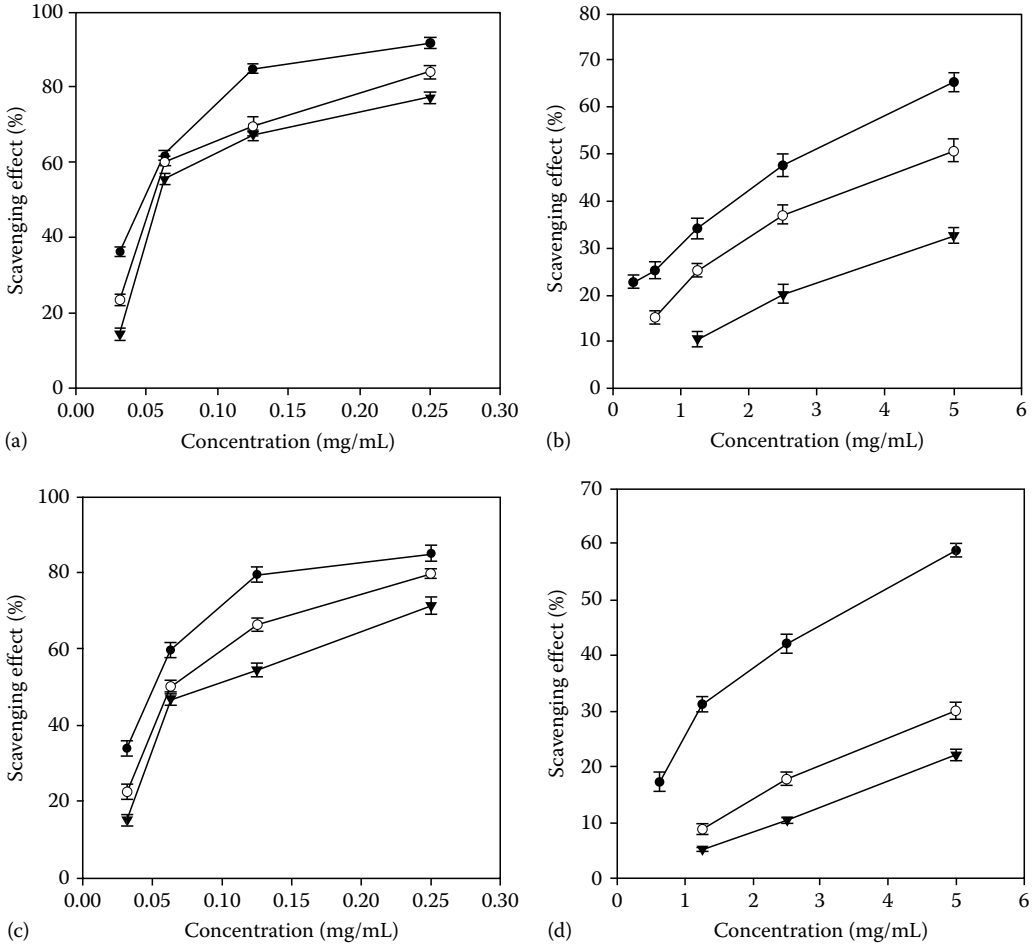

FIGURE 18.5 Hydroxyl and superoxide radical scavenging effects of chitosan derivatives derived from 90% and 50% deacetylated chitosan at various concentrations. Scavenging effect was calculated by relative ESR signal intensity compared with control ESR spectrum. (a) Hydroxyl-radical scavenging activity of 90% deacetylated chitosan; (b) hydroxyl-radical scavenging activity of 50% deacetylated chitosan; (c) superoxide-radical scavenging activity of 90% deacetylated chitosan; (d) superoxide-radical scavenging activity of 50% deacetylated chitosan. AEC (●), DMAEC (○), and DEAEC (▾). Values represent means ± SE ($n = 3$). (From Je, J.Y. and Kim, S.K. *Bioorg. Med. Chem.,* 14, 5989, 2006. With permission.)

18.4 CONCLUSIONS

Chitosan (*N*-deacetylated derivative of chitin), a naturally abundant mucopolysaccharide and its derivatives containing COSs were prepared by enzymatic and chemical modification. In addition, chitosan and its derivatives showed potent antioxidant activity. Moreover, chemical modification of chitosan can be easily provided with more powerful antioxidative compounds. Considering the above findings, it is expected that chitosan and its derivatives contained COSs would be promising candidates as potent antioxidative agents leading to their valuable use in neutraceuticals and expand their applications in Biomedicine.

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19 Effects of Chitin, Chitosan, and Their Derivatives on Human Hemostasis

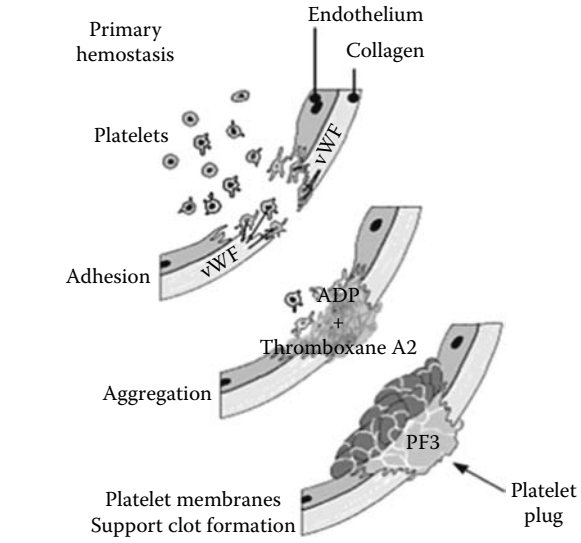
Se-Kwon Kim and Won-Kyo Jung

CONTENTS

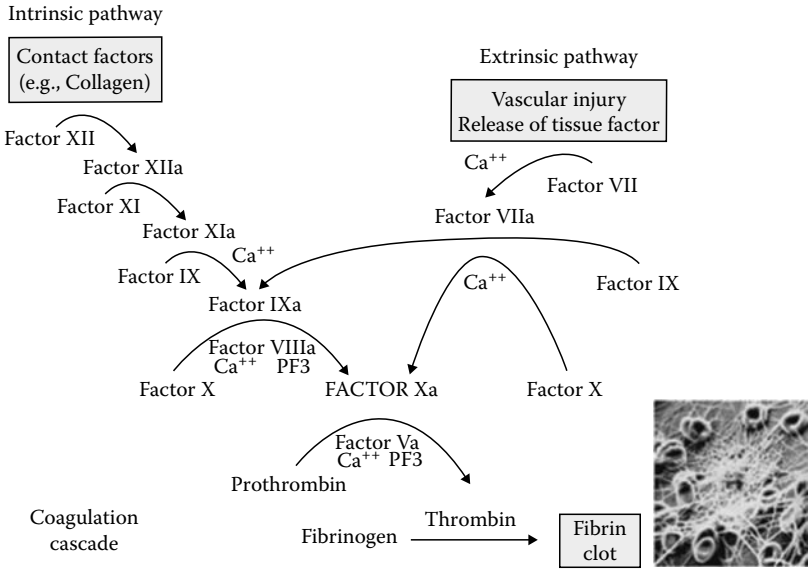
19.1 INTRODUCTION

The ability of the human body to control the flow of blood following vascular injury is paramount to continued survival. The process of blood clotting and then the subsequent dissolution of the clot, following repair of the injured tissue, is termed hemostasis. To stop bleeding, the injured vessel wall is processed by primary platelet-mediated aggregation (Figure 19.1a), a secondary blood coagulation pathway (Figure 19.1b), retraction of fibrin clot (Figure 19.1a), and fibrinolysis after vascular constriction, as described in the following.

1. The initial phase of the process is *vascular constriction*. This limits the flow of blood to the injured area.
2. Next, platelets become activated by thrombin and aggregate at the site of injury, forming a temporary, loose platelet plug (Figure 19.1a). *Platelet aggregation* is the primary process of hemostatic mechanism and is set in motion when the endothelial lining of blood vessels is denuded following vascular injury.
3. *Blood coagulation pathway*, the secondary process involves the interaction of many plasma serine proteases known as clotting factors (Figure 19.1b). These factors (Table 19.1) interact with calcium and phospholipid surface to produce a tough fibrin meshwork, which reinforces the friable platelet plug and stops bleeding until tissue repair can occur. This coagulation cascade was first described over 40 years ago (Davie and Ratnoff 1964). Being a very complex system, the theories on its intrinsic entity are still changing. However, there



(a)



(b)

FIGURE 19.1 Human blood clotting system (hemostasis). (a) Platelet-mediated primary hemostasis: Contraction of the wounded blood vessel (serotonin, thromboxane A2) → Adhesion of platelets on the blood vessel (von Willeblend factor, thromboxane A2) → Aggregation of platelets. (b) The schematic diagram of classical coagulation cascade. Coagulation is initiated by converging two pathways at the formation of FXa, which triggers the activation of thrombin (IIa) from prothrombin (II). The final step in the cascade of coagulation factors involves FXIII which cross-links fibrin molecules to a clot-stabilizing mesh over the aggregated platelets.

are no doubts of the importance of the coagulation cascade in the hemostasis leading to an endpoint of a stabilized clot.

4. *Fibrinolysis* is the final process of the hemostatic mechanism. Like coagulation, fibrinolysis is a complicated interplay between activators and inhibitors. In this process plasminogen is converted to plasmin, the fibrinolytic enzyme that lyses fibrin clot.

TABLE 19.1
Properties of Enzymes as Coagulant Factors as in Human Blood Coagulation Pathways

Property	FIIa (Thrombin)	γ-Thrombin	FVIIa	FIXa	APC	FXa	FXIa	FXIIIa	Plasmin
Localization	Plasma	Plasma	Plasma	Plasma	Plasma	Plasma	Plasma	Plasma	Plasma
Isoelectric point	7.0–7.6	—	—	—	4.4–4.8	—	—	5.2[a]	6.7–8.3
Mode of actions	Serine protease which cleaves fibrinogen to form fibrin; also responsible for activation of protein C, platelet activation, and feedback activation of the procofactors, factor V and factor VIII	Proteolyzed forms of α-thrombin which retain activity toward small substrates, factor XIII and prothrombin, but have reduced activity toward fibrinogen, protein C activation and antithrombin III binding	Enzyme component of the extrinsic factor X activating complex; also activates factor IX, thus by-passing the contact activation system	Enzyme component of the Factor Xase complex	Anticoagulant, inactivates factors Va and VIIIa	Enzyme component of the prothrombinase complex	A serine protease that converts factor IX to factor IXa	Plasma transglutaminase	Enzyme involved in fibrinolysis
Molecular weight	36,700	35,400 (β-thrombin) 34,300 (γ-thrombin)	50,000 (human)	45,000	56,200	46,000 (human)	160,000	312,000	83,000 (lys-plasmin)
Percent carbohydrate	5%	—	13%[b]	—	23%	3.0%	5%	A chain: 1%/B chain: 5%	2%

(*continued*)

TABLE 19.1 (continued)
Properties of Enzymes as Coagulant Factors as in Human Blood Coagulation Pathways

Property	FIIa (Thrombin)	γ-Thrombin	FVIIa	FIXa	APC	FXa	FXIa	FXIIIa	Plasmin
Structure	Two subunits, approximately Mr = 6,000 and 31,000	β-thrombin: three chains (A, B1, B2), disulfide link between the A and the B2 chains. γ-thrombin: four chains (A, B1, B5, B4) with a disulfide link between the A peptide and the B5 peptide	Two subunits; NH_2-terminal derived light chain (Mr = 20,000), COOH-terminal derived heavy chain (Mr = 30,000), NH_2-terminal gla-domain, two EGF domains	Two subunits, Mr = 28,000 and 17,000, NH_2-terminal gla-domain, two EGF domains	Two chains, Mr = 35,000 and 21,000, disulfide linked, NH_2-terminal gla domain two EGF domains	Two subunits, Mr = 16,200 and 29,000 (human) Mr = 16,500 and 28,800 (bovine) NH_2-terminal gla domain, two EGF domains	Two apparently identical heavy chains (Mr ~ 50,000) and two apparently identical light chains (Mr ~ 30,000) held together by disulfide bonds. Each light chain contains a catalytic domain	Tetramer ($A_2'B_2$) in the absence of calcium, two identical A chains (Mr = 71,000) each containing six free sulfhydryls and active site, two identical B subunit (Mr = 88,000) identical subunits, A (Mr = 75,000), B (Mr = 88,000)	Two subunits, Mr = 57,000 and Mr = 26,000, disulfide linked, 5 kringle domains 22 disulfide bridges, NH_2-terminal lysine
Posttranslational modification	—	—	One β-hydroxyaspartate, ten gla residues	One β-hydroxy aspartate twelve gla residues	11 gla residues (bovine), 9 gla residues (human), 1 β-hydroxyaspartate	11 gla residues, 1 β-hydroxyaspartate	—	—	—

Source: Courtesy of Haematologic Technologies, Inc., Essex Junction, VT, Research Reagents Catalog, 2004–2005.

[a] Inferred from the zymogen, factor XI.

[b] Based upon analysis of bovine factor VII.

The activation of blood coagulation as well as platelet activation are important in the pathogenesis of arterial thrombosis. These two fundamental mechanisms of thrombogenesis are closely linked *in vivo*, because thrombin, a key clotting enzyme generated by blood coagulation, is a potent platelet activator, and activated platelets augment the coagulation process. Therefore, both anticoagulants and drugs that suppress platelet function are potentially effective in the prevention and treatment of arterial thrombosis. Among major agents for anticoagulant therapy, heparin, a sulfated glycosaminoglycan consisting of chains of polysaccharides produced by mammalian mast cells, has been used extensively during the last 50 years as an anticoagulant (Hirsh 1991, Olsen and Björk 1994; Weitz 1994, Beijering et al. 1996, Weitz and Hirsh 2001). However, heparin has some well-documented problems related to its clinical application such as its inefficacy in antithrombin-deficient patients, poor bioavailability, a variable and extremely steep dose–response curve, and heparin-induced thrombocytopenia developing in some heparin recipients despite its widespread clinical use as an anticoagulant. In the past few years, many researchers have attempted to overcome some problems associated with heparin. The studies have been aimed at the development of anionic sulfated polysaccharide heparin-like substances such as dermatan sulfate, the synthetic sulfated oligosaccharides, bis-maltobionic acid amide (maltodapoh), and bis-lactobionic acid amide (aprosulate), as well as synthetic antithrombin III (AT III)-binding heparin pentasaccharides (Petitou and van Boekel 2004).

Chitin is a naturally abundant mucopolysaccharide and distributed in the shell of crustaceans, in the cuticle of insects, and also in the cell wall of some fungi and microorganisms. Chitin consists of 2-acetamido-2deoxyl-(1,4)-β-D-glucopyranose residues (*N*-acetyl-D-glucosamine units) that has intra- and inter-molecular hydrogen bonds and is a water-insoluble material resembling cellulose in its solubility. Chitosan, which is obtained by the partial deacetylation, is a natural polysaccharide comprising copolymers of glucosamine and *N*-acetylglucosamine (Illum 1998, Nunthanid et al. 2001). While chitin has a limited application because of its poor solubility and reactivity, chitosan is soluble in acetic acid and other organic solvents. Therefore, chitosan has been widely used in vastly diverse fields such as pharmaceutics, medicine, and biotechnology (Muzzarelli 1997).

However, increasing attention has recently been given to converting its oligosaccharides because of their specific biological activities such as anticoagulant activity (Vongchan et al. 2002, Park et al. 2004), antitumor activity (Suzuki et al. 1986, Suzuki 1996, Jeon and Kim 2002), immunostimulating effects (Tokoro et al. 1989, Jeon and Kim 2001), enhancing protective effects against infection with some pathogens in mice (Yamada et al. 1993), antifungal and antimicrobial activity (Allan and Hadwiger 1979, Hadwiger and Beckman 1980, Walker-Simmons et al. 1983, Hirano and Nagao 1989, Kendra et al. 1989, Jeon et al. 2001), and radical scavenging activity (Park et al. 2003). Recently, hemostatic effects of chitin, chitosan, and their derivatives have been reported on both their positive and negative interactions with the key hemostatic factors, platelet aggregation, and blood coagulation factors. We summarize hemostatic effects of chitin and chitosan, and their derivatives in this chapter.

19.2 EFFECTS ON PLATELET ACTIVATION AND AGGREGATION

19.2.1 Mechanism of Platelet Aggregation

Platelets become activated by thrombin and aggregate at the site of injury, forming a temporary, loose platelet plug (Figure 19.1a). Platelet aggregation is the primary process of hemostatic mechanism is set in motion, when the endothelial lining of blood vessels is denuded following vascular injury. Platelets, which are circulating in the blood, come into contact with the injured vessel wall and adhere to the exposed underlining collagen layer. Adherence of platelets is rapidly followed by a change in the shape of the platelets, a release reaction, and then by their aggregation to one another. The platelets change their shape from that of a disc to a spheroid with the extension of many little pseudopods on their surface. During the release reaction, these activated platelets extrude the contents of their cytoplasmic granules, realizing a number of chemical products including ADP,

serotonin, platelet factor 4, beta-thromboglobulin, fibronectin, fibrinogen, factor V, and some other compounds. The ADP released from the platelet granules is a powerful platelet aggregator and recruits other platelets into the growing platelet mass to finally form a platelet plug which temporarily arrests bleeding. During platelet aggregation or primary hemostasis, other than the release of cytoplasmic granules, platelets themselves provide phospholipid surface, which is critical for interacting with clotting factors in clotting cascade.

19.2.2 The Physiological Role of Chitin and Chitosan

Chitin and chitosan enhance platelet adhesion and aggregation. According to the experimental results by Chou et al. (2003), chitosan (MW = 50 kDa, deacetylation degree DD > 90%) dose-dependently increased the platelet aggregation (Figure 19.2) and the intracellular free Ca^{2+} rise of Fura-2-AM loaded platelets. Additionally, in the presence of FITC-labeled anti-CD41/CD61, chitosan significantly enhanced the expression of platelet glycoprotein IIb/IIIa complex assayed by a flow cytometer. It was concluded that chitosan is an effective inducer for platelet adhesion and aggregation and the mechanisms of action of chitosan may be associated, at least partly, with the increasing Ca^{2+} mobilization and enhancing expression of GPIIb/IIIa complex on platelet membrane surfaces.

Okamoto et al. (2003) reported that the effects of chitin and chitosan on platelet aggregation were evaluated (Okamoto et al. 2003). In the investigation, chitin (MW = 300 kDa, DD < 10%) and chitosan (MW = 80 kDa, DD > 80%) enhanced the release of the platelet derived growth factor-AB (PDGF-AB) in a dose-dependent manner and the transforming growth factor-b1 (TGF-b1) from the platelets, particularly, more with chitosan.

19.2.3 The Physiological Role of Chitin and Chitosan Derivatives

The effect of various sulfonated derivatives of chitosan on platelet activation was also examined by Lin and Lin (2001). In this study, the sulfonation was directly performed on the chitosan membrane surface.

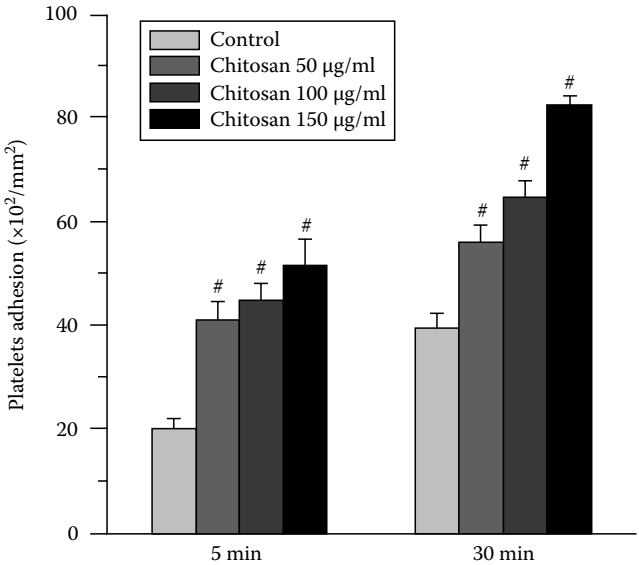


FIGURE 19.2 Enhancement of chitosan on initial and long-term platelet adhesion. Platelets were added to vehicle- (0.04% citric acid as solvent control) or chitosan-coated microtiter plates, and incubated at room temperature for 5 or 30 min. Then, the adhesion of platelets to these coated plates was measured. Results represent means ± SEM of three separate experiments, each performed in triplicate. #P < 0:001 as compared with the control, respectively. (From Chou, T.C. et al., *Biophys. Res. Commun.*, 302, 480, 2003. With permission.)

In the platelet adhesion assay, *N*,*O*-sulfated chitosan with cationic NH_3^+ groups was shown to reduce platelet adhesion and activation by ionic interactions between the platelets membrane surface and the cationic groups on the modified chitosan membrane.

The effects of chitin and chitosan derivatives on *in vitro* human platelet activation were comparatively studied by Janvikul et al. (2006). Chitin, chitosan, partially *N*-acetylated chitosan (PNAC), *N*,*O*-carboxymethylchitosan (NOCC), *N*-sulfated chitosan, and *N*-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride were tested for platelet aggregation assay. The results revealed that NOCC activated platelets most effectively.

Compared to heparin, the sulfated chitosan has been shown to possess high anticoagulant potency. Unlike heparin, sulfated chitosan does not show anti-platelet activity, which causes excessive bleeding in patients (Nishimara et al. 1998).

19.3 EFFECTS ON HUMAN BLOOD COAGULATION PATHWAY

19.3.1 Human Blood Coagulation Pathway

Blood coagulation pathway, the secondary process involves the interaction of many plasma serine proteases known as clotting factors (Figure 19.1b). These factors interact with calcium and phospholipid surface to produce a tough fibrin meshwork, which reinforces the friable platelet plug and stops bleeding until tissue repair can occur. This coagulation cascade was first described over 40 years ago (Davie and Ratnoff 1964). Being a very complex system, the theories on its intrinsic coagulation cascade are still changing. However, there are no doubts of the importance of the coagulation cascade in the hemostasis leading to an endpoint of a stabilized clot. The coagulation cascade consists of an intrinsic pathway, so named originally, because the presumed initiative components were present in blood, and an extrinsic pathway, which requires subendothelial tissue factor (TF) for its activation. The two pathways converge at the formation of FXa by activated FIX (from intrinsic pathway) and FVII (from extrinsic pathway), which leads to thrombin formation in common pathway. In the original theory, the intrinsic pathway was thought to carry the main responsibility for the initiation of the coagulation cascade. However, nowadays, it is generally accepted that the tissue factor pathway acts as a "prima ballerina" in the initiation, and the intrinsic pathway plays a more important role in the continuity of the coagulation (Luchtman-Jones and Broze 1995, Schmaier 1997). The extrinsic pathway or tissue factor pathway is activated as the disruption of a vessel wall exposes TF to circulating plasma components factor VII or VIIa (Bauer 1997). They form a complex, which proteolytically activates factors IX and X. Some of factor Xa proceeds to catalyze the conversion of prothrombin (FII) to thrombin (FIIa). Other part of Xa forms a complex with molecules called tissue factor pathway inhibitors 1 or 2 (TFPI-1, -2), which function as negative feedback controllers of the extrinsic pathway (Rapaport and Rao 1995). In addition, to sustain the coagulation process, thrombin is also formed by the intrinsic pathway, namely through the action of FIXa in concert with its cofactor VIIIa. The final step in the coagulation cascade is the formation of a stabilized fibrin clot (Figure 19.1b). Thrombin produced in the coagulation process converts soluble fibrinogen into fibrin monomers and simultaneously activates a transglutaminase FXIII, which in turn induces the covalent cross-linking of formed fibrin monomers to polymers in a transamidation reaction. In the original coagulation cascade theory, the initiative proteins in the intrinsic pathway were the high molecular kallikrein (HK), prekallikrein, and factor XII (FXII) that were found grouped together and named the "contact system," because they needed a contact with artificial, negatively charged surfaces for zymogen activation *in vitro*. The contact system was considered to lead to the activation of FXI and IX and further FX. This cascade was thought to be essential because deficiencies in the contact pathway proteins had been shown to result in prolonged activated partial thromboplastin time (APTT) (Colman et al. 1975, Saito et al. 1975, Wuepper et al. 1975). In distinction to this theory, some believe that the activation of FXI by still some unknown agents is the real initiative component for the intrinsic coagulation pathway (Schmaier 1997). Blood coagulation is inhibited at

several points by such factors as TFPI-1 and TFPI-2 (Saito et al. 1975, Broze and Miletich 1987, Rao 1987, Sprecher et al. 1994), protein C (Kisiel 1979) with its cofactor protein S (Discipio and Davie 1979, Walker 1981), and antithrombin III (Rosenberg and Damus 1973), which are all proteins produced outside of the coagulation cascade and act to prevent generalized thrombus formation.

19.3.2 The Physiological Role of Chitin and Chitosan

Chitin and chitosan has been reported to possess hemostatic or procoagulant activity tested *in vitro* or *in vivo*. Okamoto et al (2003) and Janvikul et al. (2006) showed that chitin and chitosan reduced blood coagulation time in a dose-dependent. Therefore, positively charged chitosan is more effective than chitin as a blood coagulant (Rao and Sharma 1997). Furthermore, they suggested that chitosan as a topical hemostatic agent has potential commercial value not only in intraoperative procedures but also in general topical emergencies in hospitals where the facilities are limited to managing bleeding problems as wound-healing agents.

However, Park et al. (2004) reported that chitosan process anticoagulant activity was tested *in vitro*. They hypothesized that blood coagulation factors in the presence of chitosan with a glucosamine structure might be dependent in terms of degree of deacetylation, molecular size, and other physical characters.

19.3.3 Antithrombotic Drugs with Sulfated Polysaccharides

In the early 1980s, heparin, the main drug in the field of venous thrombosis, was submitted to chemical or enzymatic fragmentation, and the resulting low molecular weight heparins became the market leaders in antithrombotics, with current world sales of about two billion dollars. At the same time, the AT-III-binding domain (ABD) of heparin was identified (Figure 19.3), and the ambitious objective of obtaining synthetic drug substances with the excellent antithrombotic properties of the complex polysaccharides has been issued.

Throughout the research and development for antithrombotic sulfated polysaccharides, the first synthetic antithrombotic pentasaccharide (Arixtra, fondaparinux sodium) was produced (Figure 19.4a). Sanofi and Organon Co. developed a synthetic analogue of this pentasaccharide. The resulting antithrombotic drug Arixtra (fondaparinux), which went on the market in the United States

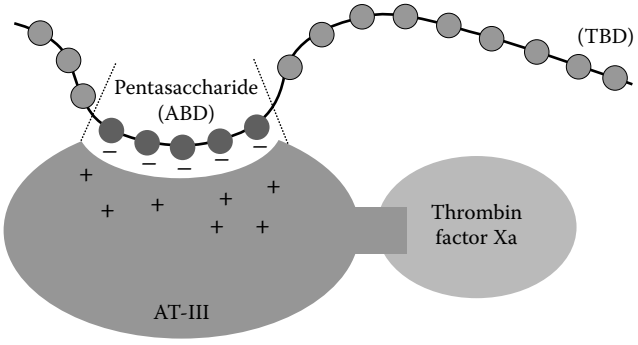


FIGURE 19.3 Heparin interacts with antithrombin III (AT-III) to bring about a conformational change in the serine protease inhibitor, thus allowing the loop with the reactive center to interact with coagulation enzymes, such as thrombin and factor Xa. To induce the conformational change in AT-III, a unique pentasaccharide domain—also called the antithrombin III binding domain (ABD)—should be present in the heparin chain. The pentasaccharide stimulates exclusively the AT-III-mediated inactivation of factor Xa (anti-Xa activity), whereas longer heparin fragments comprising both the pentasaccharide domain (ABD) and a thrombin-binding domain (TBD) are required for stimulating antithrombin activity. (From Petitou, M. and van Boeckel, C.A.A., *Angew. Chem. Int.*, 43, 3118, 2004. With permission.)

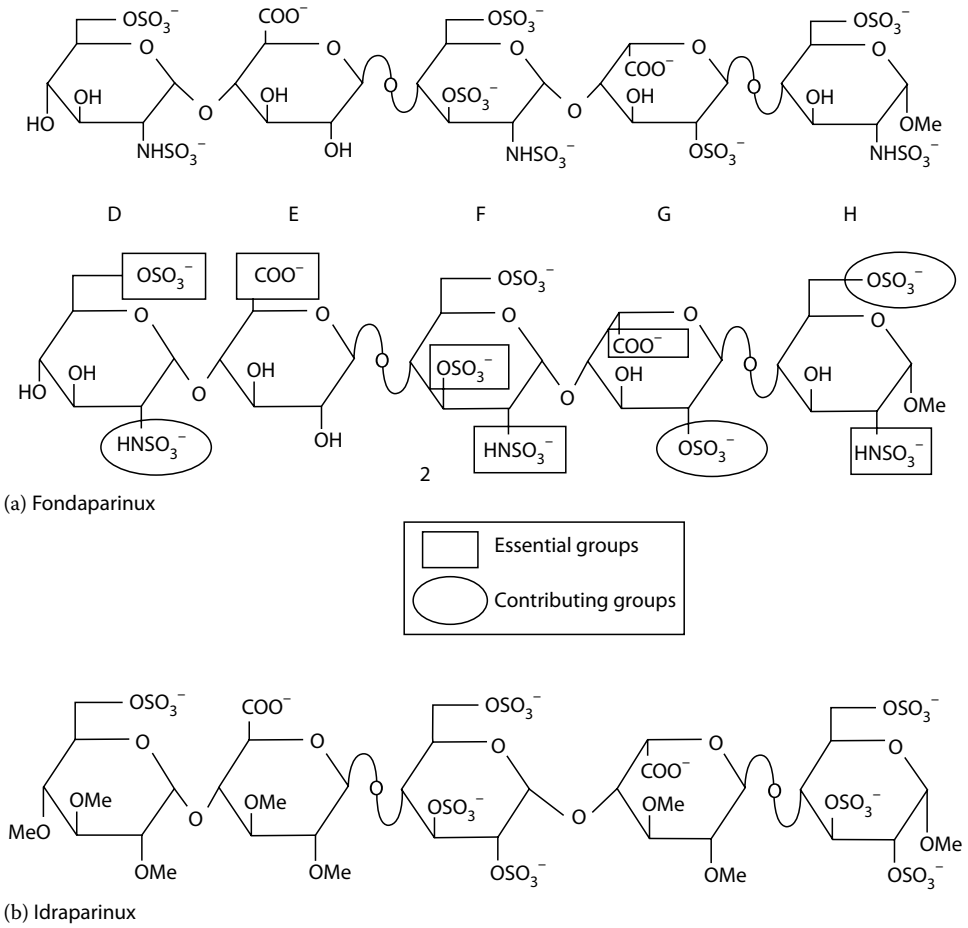


FIGURE 19.4 Structure–activity relationships of fondaparinux (a) and idraparinux (b). Analogues were synthesized that lacked one particular sulfate or carboxylate group. In this way it was established that the groups highlighted in the boxes are absolutely essential for the activation of AT-III, whereas the groups in the circles only help to increase the biological activity. (From Petitou, M. and van Boeckel, C.A.A., *Angew. Chem. Int.*, 43, 3118, 2004. With permission.)

and Europe in 2002, shows superior antithrombotic activity and brings about AT-III-mediated activity against factor Xa exclusively. Structure-based design has subsequently led to analogues with longer-lasting activity, such as idraparinux (Figure 19.4b), as well as novel conjugates and long oligosaccharides with specific anti-Xa and antithrombin activities. The new drug candidates are more selective in their mode of action than heparin and less likely to induce thrombocytopenia (Petitou and van Boeckel 2004). Recently, many experiments have been attempted to build non-toxic and more selective heparin-like structure and activity through chemical modification of basic structure of chitosan with glucosamine subunit.

19.3.4 Antithrombotic Chitosan Derivatives

Heparin, a sulfated glycosaminoglycan has been used clinically as an antithrombotic agent. It was discovered that a unique pentasaccharide domain in some heparin chains activates antithrombin III (AT-III), a serine protease inhibitor that blocks thrombin (factor IIa) and factor Xa in the coagulation cascade. However, heparin has some well-documented problems related to its clinical

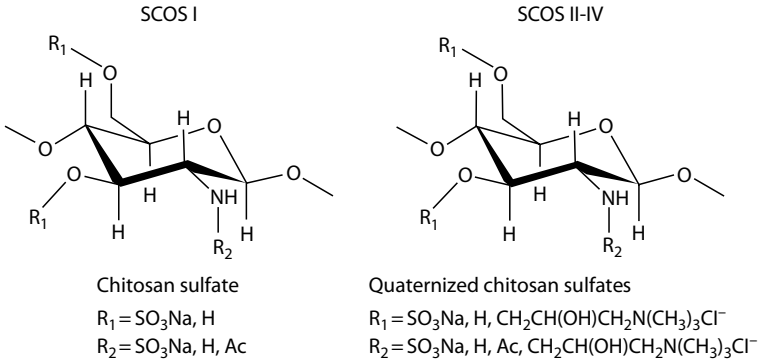


FIGURE 19.5 Chemical structures of the SCOSs with anticoagulant activity. (From Huang, R. et al., *Carbohydr. Res.*, 338, 483, 2003. With permission.)

application such as its inefficacy in antithrombin-deficient patients, poor bioavailability, a variable and extremely steep dose–response curve, and heparin-induced thrombocytopenia developing in some heparin recipients (Hirsh 1991) despite its widespread clinical use as an anticoagulant. In the past few years, many researchers have attempted to overcome some problems associated with heparin. The researches have been aimed at the development of sulfated polysaccharide heparin-like substances such as dermatan sulfate, the synthetic sulfated oligosaccharides bis-maltobionic acid amide (maltodapoh) and bis-lactobionic acid amide (aprosulate), as well as synthetic antithrombin III (AT-III)-binding heparin pentasaccharides (Petitou and van Boeckel 2004).

Compared to heparin, various sulfated chitosan oligosaccharide (SCOSs) derivatives (Figure 19.5) have been shown to possess high anticoagulant potency. In the case of SCOS I, sulfation was carried out using chlorosulfonic acid in *N*,*N*-dimethylformamide at room temperature to avoid degradation of chitosan. A higher degree of sulfation was shown to be beneficial for the anticoagulant activity, with respect to thrombin time. The arrangement of sulfate groups was found to have tremendous influence on the anticoagulation process, for example, C-6 sulfate group was a key requirement, as its desulfation led to loss of activity (Nishimara et al. 1998).

To prepare *N*-propanoyl-, *N*-hexanoyl-, and *N*,*O*-quaternary substituted chitosan sulfate with the similar structure of heparin, the chemical modification of chitosan sulfate was carried out by Huang et al. (2003). The propanoyl (SCOS II) and hexanoyl (SCOS III) groups increased the thromboplastin time (APTT) activity, and the propanoyl groups also increased the thrombin time (TT) anticoagulant activity slightly, while the *N*,*O*-quaternary chitosan sulfate (SCOS IV) showed only a slight TT coagulant activity.

These results illustrated that anticoagulant activity of sulfated polysaccharides generally results from the interaction between the negatively charged sulfated groups and positively charged peptide sequences of AT-III as an endogenous inhibitor against thrombin (factor IIa) and factor Xa in the coagulation cascade, and *N*-acetyl groups in glucosamine unit improve the anticoagulant activity.

19.4 CONCLUSION

Chitin, chitosan, and their derivatives possess various biological activities and have a considerable potential to be utilized in number of medicinal applications. Considering the emerging physiological importance of natural polysaccharides, many researchers have demonstrated that through organic synthesis and rational design heparin-like compounds can be prepared, whereby not only the AT-III-mediated anti-Xa and antithrombin activity can be tuned precisely but also the half-life in circulation, and nonspecific interactions with other basic proteins can be diminished (Petitou and van Boeckel 2004). These anticoagulants have been used as convenient tools for exploration

of the complex mechanisms of the coagulation cascade. Coincidentally, the importance of research seeking anticoagulants has also arisen from the need for therapeutic agents that might help cure hemophilia (Jung et al. 2002).

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20 Antihypertensive Actions of Chitosan and Its Derivatives

Jae-Young Je and Chang-Bum Ahn

CONTENTS

20.1 INTRODUCTION

The naturally occurring biopolymer, chitin, is a well-known mucopolysaccharide abundantly distributed in the shell of crustaceans, in the cuticle of insects, and also in the cell walls of some fungi and microorganisms. Chitin consists of 2-acetamido-2-deoxy-(1–4)-β-D-glucopyranose residues (*N*-acetyl-D-glucosamine units) that has intra- and intermolecular hydrogen bonds and is a water-insoluble material resembling cellulose in its solubility. Chitosan is an *N*-deacetylated derivative of chitin and consists of 2-amino-2-deoxy-(1–4)-β-D-glucopyranose residues (D-glucosamine units). Chitosan has many interesting properties such as biocompatibility, biodegradability, less toxic nature, and versatile chemical and physical properties that make it attractive for a wide variety of applications in many fields such as food (Lin et al. 2009), cosmetics (Majeti and Kumar 2000), biomedicine (Felt et al. 1998), agriculture, and wastewater management (Kim et al. 2007). Due to its versatile biological activities such as antibacterial activity (Allan and Hadwiger 1979, Hirano and Nagao 1989, Jeon and Kim 2000, Jeon et al. 2001), hypocholesterolemic activity (Hirano et al. 1990), antitumor activity (Sugano et al. 1992), immumostimulating effect (Jeon and Kim 2001), and antioxidant activity (Park et al. 2004), chitosan has been under much attention to develop as new physiological bioactive materials. In spite of its unique biological aspects, the water-insoluble property is a major limiting factor for its wide application. Moreover, biofunctionalities of chitosans are highly related to its molecular weight (MW) and the degree of deacetylation (DD). To this end, recent progress in basic and application studies about chitosan chemistry has been achieved, which develops ways to improve not only water-soluble property but also biological activities by using both chemical and enzymatic modifications. This chapter provides an overview of antihypertensive effects of chitosan and its derivatives prepared by enzymatic and chemical modifications.

20.2 RENIN–ANGIOTENSIN SYSTEM: DEVELOPMENT OF ANTIHYPERTENSIVE AGENTS

The renin–angiotensin system (RAS) plays a pivotal role in the control of blood pressure and the pathophysiology of cardiovascular diseases such as congestive heart failure and hypertension (Brunner et al. 1972, Waeber et al. 1986). Renin, also known as angiotensinogenase, is a circulating enzyme secreted by the granular cells of juxtaglomerular apparatus in the kidney. Renin belongs to the class of aspartyl proteases (Inagami et al. 1974) and has high substrate specificity, and catalyzes the hydrolysis of only one naturally occurring substrate, angiotensinogen (Stanton 2003). Renin controls the first and rate-limiting step enzyme in RAS and cleaves the Leu10-Val11 peptide bond of plasma angiotensinogen and releasing the decapeptide angiotensin I (Figure 20.1), which is further converted by soluble or endothelial cell–associated angiotensin-converting enzyme (ACE) to angiotensin II (Ang II), a powerful vasoconstrictor that has been identified as a major factor in hypertension (Peach 1997). ACE belongs to the class of zinc proteases and is located in the vascular endothelial lining of lungs. ACE acts as an exopeptidase that cleaves dipeptides from the C terminus of various oligopeptides (Curtiss et al. 1978). ACE is also involved in the inactivation of bradykinin, a potent vasodilator.

Hypertension is one of the most common cardiovascular diseases and has become a worldwide problem of epidemic proportions, which presents in 15%–20% of all adults. Excessive RAS activity is believed to increase blood pressure by the overproduction of Ang II. Moreover, hypertension is the most common serious chronic health problem because it carries a high risk factor for arteriosclerosis, stroke, myocardial infarction, and end-stage renal disease (Zhang et al. 2006). Since the discovery of ACE inhibitors in snake venom, many studies have been attempted in the synthesis of ACE inhibitors such as captopril, enalapril, alacepril, and lisinopril, which are currently used extensively in the treatment of essential hypertension and heart failure in humans (Ondetti 1997). Extensive clinical studies using ACE inhibitors have shown the importance of RAS in blood pressure regulation and led to the therapeutic application of ACE inhibitors in hypertension (Rahuel et al. 2000). More recently, with the model development of the catalytic structure of ACE, specific inhibitors that can bind to the enzyme active site have been developed. Moreover, antagonists of the Ang II receptor were developed to interfere more specifically with RAS but increased angiotensin peptide levels. Furthermore, Ang II can be produced through the hydrolysis of Ang I by chymase in the heart, which can reduce the efficacy of ACE inhibitors. Since renin is a rate-limiting step in the RAS and has a specific substrate (angiotensinogen), renin inhibition is thought to be an attractive target for antihypertension strategy without any side effects. Most of the renin inhibitors are mainly peptide analogs that are derived from substrate preprorenin, pepstatin, and related analog,

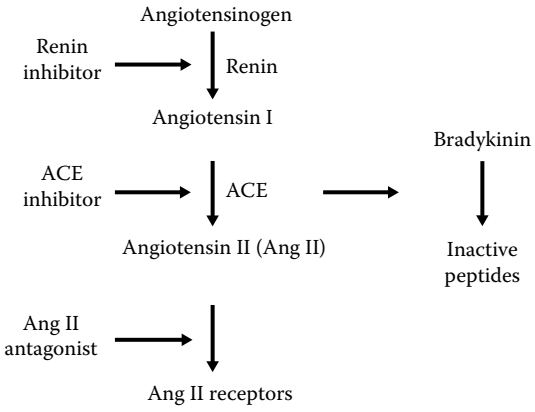

FIGURE 20.1 The renin–angiotensin cascade. ACE, angiotensin converting enzyme; Ang II, angiotensin II.

phospholipids, and renin antibody (Yuan et al. 2006). However, their poor pharmacokinetic properties (poor absorption and rapid elimination) following oral administration in human have made them unattractive as clinical candidates. Hence, finding new kinds of RAS inhibitors would be a good challenge in the development of antihypertensive agents.

20.3 ANTIHYPERTENSIVE ACTIVITY

Chitosan is known to have many biological activities, which are affected by MW or chain length and DD. Therefore, desired properties of chitosan or its derivatives that are necessary to exhibit different biological activities can be obtained by chemical and enzymatic modification. In this chapter, antihypertensive actions of chitosan and its derivatives are discussed.

20.3.1 Chitosan

Several factors affect the increment of blood pressure in humans; among them sodium chloride was recognized as a major factor. A high level of sodium chloride in the diet has been shown to increase the blood pressure of human and animal models of hypertension (Boon and Aronson 1985). Several investigators noted that the ingestion of nonchloride sodium salts did not cause an elevation of the blood pressure but the ingestion of chloride salts has been shown to be associated with the elevation of the blood pressure. These results indicate that chloride ion plays a key role in the elevation of the blood pressure in human and animal models of hypertension.

Okuda et al. (1997) studied the effect of dietary fibers on the hypertensive action of NaCl by the administration of a high-salt diet containing chitosan or alginic acid to normotensive rats and SHRSP (stroke-prone spontaneously hypertensive rats) for 40 days. The addition of chitosan to the high-salt diet resulted in a significantly lower systolic blood pressure (SBP) than that with the addition of alginic acid in both groups (Figure 20.2). Further, serum ACE was significantly reduced in SHRSP fed with the high-salt diet containing chitosan, and serum chloride ion was lower in the normotensive rats fed with the high-salt diet containing chitosan.

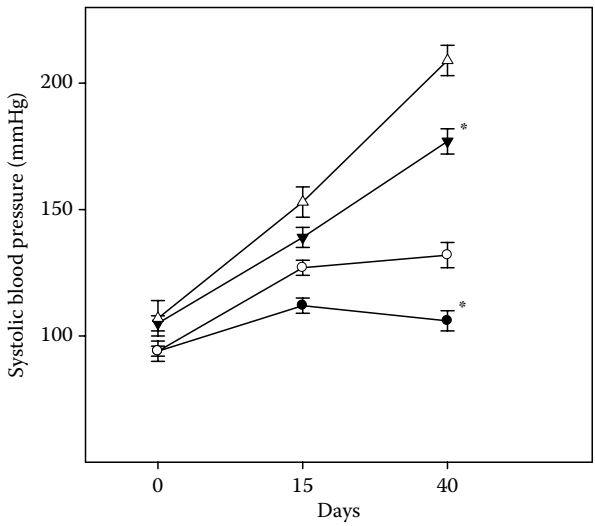

FIGURE 20.2 Changing of blood pressure by intake of high salts and dietary fibers in rats. ○, ●, normotensive rats; △, ▲, SHRSP; ○, △, alginic acid diet; ●, ▲, chitosan diet. (From Okuda, H. et al., *J. Chitin Chitosan*, 2, 49, 1997.)

In humans, the high-salt diet increased the SBP and serum ACE activity and chloride concentration after 1 h, and the oral administration of chitosan inhibited these increases. The cationic amino groups on chitosan may form polyelectrolyte complexes with negatively charged chloride ions, thus reducing both chloride ion and ACE activity, which is stimulated by chloride ions. Despite its beneficial action as a antihypertensive, the chelation of some trace metal ions from the dietary sources makes it unfavorable for human nutrition.

20.3.2 Chitooligosaccharides

Although chitosan is known to have important functional properties in many areas, their poor solubility is a major limiting factor for their application as nutraceuticals. Therefore, chitosanase-like enzymes are required to break down chitosan to small chain molecules for easy adsorption in human intestine. Two kinds of methods for the production of chitooligosaccharides (COSs) such as chemical and enzymatic production are now available. In the case of the chemical method using acid hydrolysis, COSs obtained low yields and a large amount of monomeric D-glucosamine units. Furthermore, COSs prepared by the chemical method may not be suitable for human consumption because of the possibility of the production of toxic compounds during hydrolysis (Uchida et al. 1989). Therefore, enzymatic hydrolysis for the production of COSs from chitosan has become a more preferred method during the past few decades. COSs had also been developed into physiological bioactive substances because COSs are not only water soluble but also possess versatile biological activities such as antitumor (Jeon and Kim 2002), immune-stimulating (Tokoro et al. 1988), antioxidant (Je et al. 2004), and antimicrobial activities (Jeon et al. 2001).

The effect of COSs with regard to ACE inhibition and antihypertension in spontaneously hypertensive rats (SHRs) was examined (Hong et al. 1998). Among COSs such as monomer, dimer, trimer, tetramer, pentamer, and hexamer used in ACE inhibition assay, the trimer showed the highest ACE inhibition with IC_{50} of 0.9 μM. A single oral dose of trimer with similar dose level as captopril, a known strong ACE inhibitor, was given to 8- or 21-week-old SHRs; the blood pressure reduction of both SHRs in 4 h were 27 ± 4.8 and 36 ± 4.3 mmHg, respectively. Biological activities of COSs had been shown to be greatly dependent on their DD and MW. Therefore, to identify the antihypertensive activity of COSs, it is necessary to prepare COSs with different DD and MW values. Park et al. (2003) prepared hetero-COSs from partially different deacetylated chitosans by enzymatic hydrolysis. Partially deacetylated chitosans, 90%, 75%, and 50% deacetylated chitosans, were prepared from crab chitin. The following nine kinds of hetero-COSs with relatively high MW (5,000–10,000 Da: 90-HMWCOSs, 75-HMWCOSs, and 50-HMWCOSs), medium MW (1,000–5,000 Da: 90-MMWCOSs, 75-MMWCOSs, and 50-MMWCOSs), and low MW (below 1,000 Da: 90-LMWCOSs, 75-LMWCOSs, and 50-LMWCOSs) were prepared using an ultrafiltration membrane bioreactor system. ACE inhibitory activity was increased with decreasing deacetylation of COSs, and 50-MMWCOSs exhibited the highest ACE inhibitory activity (Table 20.1). These results suggest that ACE inhibitory activity of hetero-COSs is dependent on the DD as well as the molecular size.

Surely, ACE is a major factor increasing the blood pressure by means of the production of Ang II, a powerful vasoconstrictor. However, Ang II can also be produced by chymase in the heart; therefore, the efficacy of ACE inhibitors for hypertension can be reduced. Recently, to overcome this problem, the development of renin inhibitors has been given much attention because renin has only one specific substrate, angiotensinogen, and a rate-limiting step in the RAS. Renin inhibition of COSs was also evaluated by Je and coworker. They prepared six kinds of COSs with different DD and MW values using an ultrafiltration membrane reactor. COSs suppressed the renin activity, and 90-COSs prepared from 90% deacetylated chitosan had higher potent renin-inhibitory activity than that of 50-COSs prepared from 50% deacetylated chitosan (Figure 20.3). In addition, 90-MMWCOS (MW: 1000–5000 Da) exhibits the highest activity with IC_{50} value of 0.51 mg/mL. These results also indicate that DD and MW of COSs are important factors exhibiting renin inhibition. Although

TABLE 20.1
ACE Inhibitory Activity of Hetero-COSs with Different Degrees of Deacetylation and MW

Hetero-COSs	IC_{50} (mg/mL)
90-HMWCOS	2.48 ± 0.35^{b}
90-MMWCOS	2.49 ± 0.21^{b}
90-LMWCOS	2.87 ± 0.37^{b}
75-HMWCOS	3.19 ± 0.36^{c}
75-MMWCOS	3.14 ± 0.23^{c}
75-LMWCOS	> 3.2
50-HMWCOS	1.59 ± 0.31^{a}
50-MMWCOS	1.22 ± 0.13^{a}
50-LMWCOS	1.61 ± 0.28^{a}

Source: Park, P.-J. et al., *J. Agric. Food Chem.*, 51, 4960, 2003. With permission.

[a–c] The values with different subscripts indicate significant difference ($P < 0.05$).

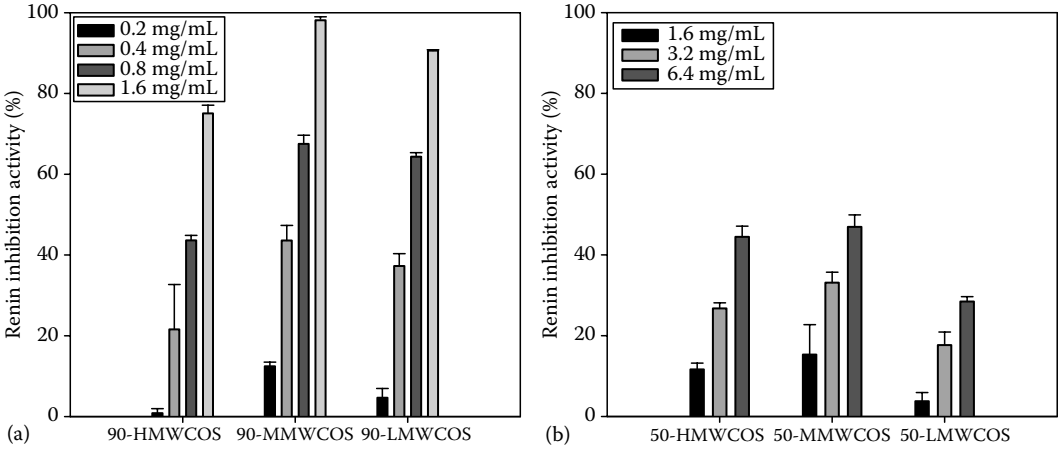

FIGURE 20.3 Human recombinant renin-inhibitory activities of COSs tested with their different DD and MW values. Results are mean ± SE of three independent experiments; 90-HMWCOS (5,000–10,000 Da), 90-MMWCOS (MW 1,000–5,000 Da), 90-LMWCOS (MW < 1,000 Da), 50-HMWCOS (MW 5,000–10,000 Da), 50-MMWCOS (MW 1,000–5,000 Da), and 50-LMWCOS (MW < 1,000 Da). (From Park, P.-J. et al., *Bioorg. Med. Chem. Lett.*, 18, 2471, 2008. With permission.)

the inhibition mode of COSs toward renin and ACE revealed different DD and MW values, COSs may be useful as a potential antihypertensive functional ingredient.

20.3.3 Chitosan Derivatives

Chitosan derivatives were developed to improve not only biological activities but also water-soluble property, because the water-insoluble property was a major limiting factor for industrial application in spite of its unique biological aspects. The improvement of structural properties of chitosan for a

particular application can be easily brought about by chemical modification. Several chitosan derivatives with antihypertensive activity were reported. Huang et al. (2005) prepared carboxylated COS to obtain specific structural features similar to captopril. The ACE inhibitory activity of carboxylated COS was increased compared to native COS, and the inhibition activity was also enhanced with increased substitution degree. They also demonstrated that the substitution of positively charged quarternized amino groups to COS effected a negative impact on ACE inhibition. In addition, aminoderivatized chitosans also have proven their ability to serve as ACE inhibitors (Je et al. 2006). Aminoethyl group was grafted onto chitosan with different DD values at the C-6 position, because the major factor involved in biological characteristics of chitosan is the amino group at C-2 position. The ACE inhibition of aminoderivatized chitosan revealed that AEC50 (aminoethyl-chitosan with 50% DD) exhibited higher ACE inhibition with IC_{50} of 0.038 μM than that of AEC (aminoethyl-chitosan with 10% DD) and AEC90 (aminoethyl-chitosan with 90% DD). This activity was superior to native chitosan and was dependent on their DD. In addition, AEC50 effectively decreased SBP on SHRs (Figure 20.4). The proposed mechanism of ACE inhibition by chitosan and their derivatives revealed that they can easily interact with the zinc ion of the active site, and the OH or NH_2 group could interact with the hydrogen of the enzyme-binding site by hydrogen bonding (Hong et al. 1998). It was also shown that the introduction of aminoethyl group at the C-6 position might increase the ability to form hydrogen bonding. Furthermore, AEC50 has exactly a half of free amino and acetyl groups (i.e., carbonyl group) in two pyranose residues, and it may be adequate to interact via

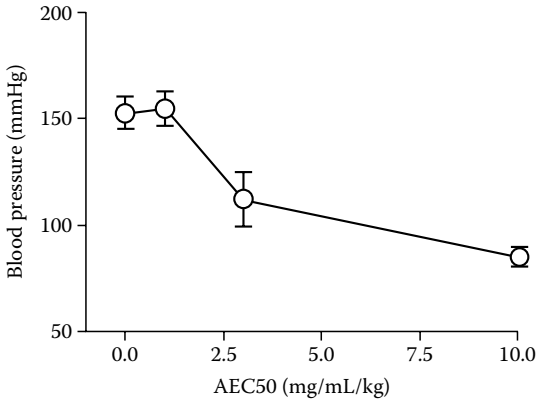

FIGURE 20.4 The change in SBP of SHRs after the administration of AEC50 (n = 5). (From Young, J.J., et al., *Biopolymers*, 83, 250, 2006. With permission.)

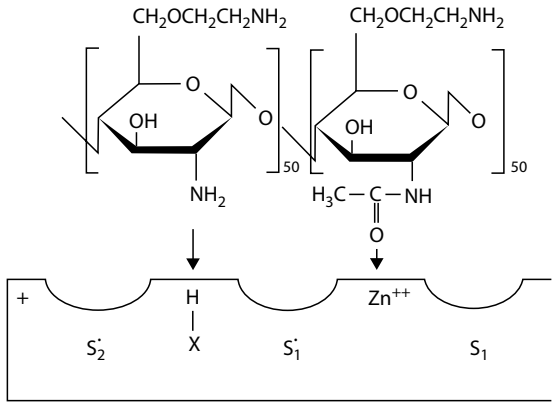

FIGURE 20.5 The hypothesized interaction between AEC50 and obligatory site of ACE. (From Young, J.J., et al., *Biopolymers*, 83, 250, 2006. With permission.)

hydrogen bonding and chelating Zn^{2+} in ACE active sites (Figure 20.5). These may be the reason for higher ACE inhibitory effects of AEC50 than that of AEC90 and AEC.

20.4 CONCLUSIONS

Chitosan and its derivatives produced by enzymatic and chemical modification have become popular biomolecules during the past few decades because of their unique biological aspects. In addition, they also showed high potential antihypertensive activity. Moreover, the chemical modification of chitosan can easily provide more powerful antihypertensive compounds. Therefore, it is expected that these biomolecules would be promising candidates as antihypertensive agents.

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21 Anticancer Activity and Therapeutic Applications of Chitosan Nanoparticles

Hang T. Ta, Dave E. Dunstan, and Crispin R. Dass

CONTENTS

21.1 INTRODUCTION

Chitosan is an amino polysaccharide obtained by alkaline deacetylation of naturally abundant chitin, a cellulose-like polymer found in fungal cell walls, yeast, lower plants, and the exoskeletons of arthropods such as insects, crabs, shrimps, lobsters, and other vertebrates. Chitin and chitosan have found wide application in a variety of areas such as paper production, textiles, metal chelation, food additives, antimicrobial agents, adhesives, and other industrial applications (Lin-Gibson et al. 2003). Due to their biodegradable, biocompatible, and antibacterial properties, they also play very important roles in medicine and pharmaceutics. Especially, chitosan is known to have immuno-enhancing effects, antitumor activities, and increased protective effects against infection caused by certain pathogens. Here we review the anticancer activities of chitosan and its use as the delivery systems for drugs and bioactive agents in cancer treatment. The application of chitosan-based nanoparticles in cancer diagnosis is also presented.

21.2 ANTICANCER ACTIVITIES OF CHITOSAN

Chitosan is well known as a pH-dependent cationic, nontoxic, antibacterial, easily bioabsorbable, biodegradable, biocompatible (Chandy and Sharma 1990, Hirano et al. 1990), and mucoadhesive biopolymer (Henriksen et al. 1996, He et al. 1998). It also possesses lots of other biological properties such as promoting wound healing (Ueno et al. 2001), anti-infection activity, and antacid and antiulcer activities that prevent or weaken drug-induced irritation in the stomach (Kumar 2000).

Besides these good characteristics, chitosan has also shown a growth-inhibition effect on tumor cells (Carreno-Gomez and Duncan 1997) and an inhibition of tumor-induced angiogenesis and tumor metastasis (Murata et al. 1991). The intratumoral administration of chitosan compounds alone was shown to promote antitumoral effects in metastatic breast cancer models (Chen et al. 1997). Chitosan was also found to activate macrophages into cytotoxic macrophages and suppressed Meth-A tumor growth in Balb/c mice (Nishimura et al. 1984). Oligosaccharides consisting of the two units that compose chitosan, *N*-acetyl-D-glucosamine and D-glucosamine, were shown to inhibit the growth of Meth-A solid tumors transplanted in mice when administered systemically (Tokoro et al. 1988). It was proposed that increased sequential production of lymphokines IL-1 and IL-2 caused the antitumor effect through the proliferation of cytolytic T-lymphocytes. Murata et al. suggested that chitosan directly inhibits tumor cell proliferation by inducing apoptosis (Murata et al. 1989). Hasegawa also reported that chitosan induced apoptosis of bladder tumor cells via caspase-3 activation (Hasegawa et al. 2001). In addition, it was reported by Guminska et al. that chitosan inhibited Ehrlich ascites tumor (EAT) cell growth by diminishing glycolysis, that is, aerobic lactate formation, thus decreasing glucose uptake and ATP level in the tumor intact cells. However, chitosan was not inhibitory on the glycolytic activity of mouse normal liver and muscle supernatants (Guminska et al. 1996). In vivo, chitosan is phagocytized by macrophages (Ono et al. 2002) and slowly degraded enzymatically by lysozyme through the hydrolysis of acetylated residues (Muzzarelli 1993). Recently, it was reported that chitosan oligosaccharides (COS) suppressed the metastasis of MDA-MB-231 human breast cancer cells (Nam and Shon 2008). This study showed the concentration-dependent decrease of cell migration and invasion with increasing concentration of COS. It was also found that COS notably downregulated the secretion, activity, and protein levels of MMP-9, a marker for cancer cells with a higher metastatic potential. It has been suggested that MMPs were involved in breast cancer invasion and metastasis. These results demonstrate that COS inhibits processes that lead to the metastasis of breast cancer cells. Chitosan is not the only polysaccharide possessing a tumor-inhibitory effect. Certain polysaccharides extracted from an edible mushroom were also found to have in vivo antitumor activity against sarcoma 180, as reported by Chihara et al. (1969). Kodama et al. (2002) also reported that polysaccharides extracted from *Grifola frondaosa* mushroom could activate lymphocyte and macrophage responses and demonstrated *in vivo* antitumor effects.

21.3 CHITOSAN-BASED SYSTEMS AS CARRIERS OF ANTICANCER DRUGS

The principal modes of cancer management are surgery, radiotherapy, and chemotherapy (WHO 2006). Recently, hormonal therapy and immunotherapy are increasingly being used as well, but their applications are limited for a few cancer types such as breast neoplasia (Wong et al. 2007). Chemotherapy, the use of cytotoxic drugs to kill cancerous cells, remains the most common approach for cancer treatment. Generally, cytotoxic drugs are highly toxic but poorly specific, and do not differentiate between normal and cancer cells. Therefore, conventional chemotherapy administration or systemic administration has been shown to produce side effects. Most of the drug content is released soon after administration, causing drug levels in the body to rise rapidly, peak, and then decline sharply, leading to unacceptable side effects at the peaks and inadequate therapy at the troughs (Kumar 2000). Due to the short duration of action, repeated injections are often required, which can lead to the exacerbation of side effects and inconvenience, and can unfortunately lead to less patient compliance. In systemic administration, cytotoxic drugs are extensively transported to the whole body, therefore, only a small fraction of the drugs reach the tumor site and other healthy organs or tissues can be affected or damaged by the nonspecific action of the cytotoxic agents (Wong et al. 2007). Due to these obstacles, controlled and targeting or localized release technology has been replacing systemic administration and has some potential for cancer treatment. Due to its antibacterial, biocompatible, biodegradable (Chandy and Sharma 1990, Hirano et al. 1990), and

mucoadhesive properties (Henriksen et al. 1996, He et al. 1998), chitosan has been widely studied and formulated in anticancer drug delivery systems.

21.3.1 Injectable In Situ Chitosan Hydrogels for Localized and Controlled Release of Therapeutics

Sustained-release injectable formulations are basically designed as microparticulates (microcapsules or microspheres), implants, or gel systems (Martini and Lauria 2003). Drugs are commonly loaded into microspheres via a passive absorption method whereby microspheres are added to drug solution. Microspheres swell in solution and the drug molecules enter the gel matrix (Sinha et al. 2004). However, the efficiency of this loading method for cytotoxic drugs is limited and a high loading capacity is unattainable. An implant requires surgery to insert it near the tumor site, which adds to the costs and the risks of this system. These problems have oriented research toward injectable in situ gelling formulations (Ruel-Gariepy et al. 2000). Injectable in situ chitosan hydrogels that have been trialed in the treatments of cancers are summarized in Table 21.1. They can be injected into the body via the two main routes of administration, including intratumoral and subcutaneous administration next to the tumor.

21.3.1.1 Photocrosslinkable Azide–Chitosan–Lactose Hydrogel

The photocrosslinkable azide–chitosan–lactose (Az–CH–LA) hydrogels were obtained by introducing photo-reactive azide groups onto chitosan chains to provide the ability to form a gel through cross-linking azide groups with amino groups under the presence of ultraviolet light (UV) irradiation (Ono et al. 2000). Lactose moieties were also added into chitosan to obtain much better water-soluble chitosan at neutral pH. In the study of Obara et al., Az–CH–LA hydrogels containing drug were administered intratumorally or locally (Obara et al. 2005). Az–CH–LA solution containing paclitaxel was subcutaneously injected beneath the tumor using an 18G needle and disposable syringe. Then a tip of optical fiber was inserted into the Az–CH–LA solution through the needle pore and UV laser irradiation was performed for 30 s to let the Az–CH–LA solution convert to insoluble hydrogel. Both the administration of Az–CH–LA hydrogel and only paclitaxel reduced subcutaneous-induced tumor growth of LLC cells within 7 days, and then they gradually lost their inhibitory activity. Surprisingly, Az–CH–LA hydrogel more strongly inhibited the growth of tumor compared to paclitaxel only; especially in the later days the difference increased.

The incorporation of paclitaxel in Az–CH–LA hydrogel resulted in the strongest inhibition of tumor growth compared to only paclitaxel and the blank Az–CH–LA hydrogel administration. Its inhibitory effect lasted for 14 days, and subsequently the tumor in almost all the mice grew again. This may be due to the lower release of paclitaxel from hydrogel matrix after time, which is consistent with the results obtained from in vitro release study. Applying a new paclitaxel-incorporated Az–CH–LA hydrogel 10 days after the first application resulted in an additional antitumor effect. After the first 14 days of administration, approximately 90% of the subcutaneously injected Az–CH–LA hydrogels in mice were biodegraded (Obara et al. 2005).

The study of Obara et al. also showed that the application of paclitaxel-incorporated Az–CH–LA hydrogel induced significant necrosis on tumor tissue and strongly inhibited angiogenesis in tumors (Obara et al. 2005). The application of free paclitaxel did not cause necrosis to tissues, and weakly inhibited tumor vascularization. Minor necrotic tumor tissue was induced by the Az–CH–LA hydrogels. The application of hydrogels also showed an intermediate effect on anti-angiogenesis. These results suggested that without a hydrogel carrier, paclitaxel molecules diffused so quickly from the injected site that they were unable to induce an antitumor effect.

TABLE 21.1
Injectable In Situ Chitosan Hydrogels in Cancer Treatments

Cancer Type	Hydrogel System	Method to Induce Gelation	Therapeutic Agent	Route of Administration	Effect	Reference
Lung cancer	Azide–chitosan–lactose (Az–CH–LA)	UV irradiation	Paclitaxel	Subcutaneously injected beneath the tumor	• Tumor-growth inhibition • Tumor-angiogenesis inhibition	Obara et al. (2005)
Breast cancer	Chitosan/β-glycerophosphate (C/GP)	Temperature	Paclitaxel	Intratumoral injection	• Tumor-growth inhibition • Tumor-recurrence prevention	Ruel-Gariepy et al. (2004)
Fibrosarcoma	Chitosan/β-glycerophosphate (C/GP)	Temperature	Camptothecin	Intratumoral injection	• Tumor-growth inhibition	Berrada et al. (2005)
Cervical cancer	Chitosan/β-glycerophosphate (C/GP)	Temperature	Doxorubicin and vaccinia virus-based vaccine expressing Sig/E7/LAMP-1 (Vac-Sig/E7/LAMP-1)	Intratumoral injection	• Tumor-growth inhibition	Han et al. (2007)
Mucin-production-associated cancers	Chitosan/glyceryl monooleate (C/GMO)	pH	Paclitaxel	—	—	Jauhari and Dash (2006)
Osteosarcoma	Chitosan/dibasic orthophosphate	Temperature	Doxorubicin and gene encoding pigment epithelium-derived factor	Subcutaneously injected next to the tumor	• Tumor-growth inhibition • Metastasis - development inhibition	Ta et al. (2009c,b)

21.3.1.2 Chitosan/β-Glycerophosphate Hydrogels

Chitosan/β-glycerophosphate (C/GP) hydrogels were formed by the neutralization of a chitosan solution with a polyol counterionic dibase salt such as β-glycerophosphate (Ruel-Gariepy et al. 2004). C/GP is a thermosensitive solution that is liquid at room temperature and solidifies into a white hydrogel at body temperature. C/GP solutions containing paclitaxel, camptothecin, or doxorubicin (Dox) were injected intratumorally (IT) (Ruel-Gariepy et al. 2004, Berrada et al. 2005, Han et al. 2007).

The local delivery of paclitaxel from the C/GP gel system injected intratumorally in EMT-6 murine mammary tumors (breast cancer) implanted subcutaneously on Balb/c mice showed that one intratumoral injection of the thermosensitive hydrogel containing paclitaxel was as efficious as four intravenous injections of Taxol in inhibiting the growth and recurrence of tumors but in a less toxic manner (Ruel-Gariepy et al. 2004). The efficacy of the treatment was demonstrated in two separate studies representing two stages of tumor growth. To investigate the ability of palitaxel–C/GP gel on tumor growth inhibition, the treatment was initiated when the tumors reached a volume of 30 mm^3. Treatment group received one injection of C/GP solution containing paclitaxel intratumorally. Control groups included saline-injected, paclitaxel IV (Taxol)-injected, and C/GP-injected groups. On day 17 of treatment, the saline-treated tumors grew to about 9 times their original size, whereas the other groups showed only approximately 5.5 times increase, which represented 38%–40% growth inhibition. Tumor growth inhibition in group received four paclitaxel injections and that in group received one paclitaxel–C/GP injection was similar.

To investigate the ability of palitaxel–C/GP gel on tumor recurrence prevention, the treatment was initiated on the fourth day of tumor growth when the tumors were very small, which mimicked a population of cancer cells remaining after primary tumor surgical excision (Ruel-Gariepy et al. 2004). Treatment groups and three control groups were treated as in the first study. After 17 days, the saline-treated tumors grew to about 18.5 times their original size, while C/GP-treated tumors grew 12 times. Both Taxol-treated and paclitaxel–C/GP-treated group showed around 5.5 times increase in tumor size. All tumors demonstrated some level of necrosis. Tumors from saline-treated group demonstrated the lowest necrotic proportions, while those from C/GP groups and Taxol-treated group showed much larger percentages of necrotic regions. During the first 6–7 days of treatment, the mice receiving Taxol displayed weight loss, while the weight of mice receiving paclitaxel–C/GP was the same as saline-treated mice. At the end of treatment (17 days), C/GP material was not found in all treated tumors. This may be because of either gel degradation or migration following breakup of the gel over time.

The effectiveness of using the C/GP systems to locally deliver high doses of camptothecin to a RIF-1 fibrosarcoma mouse model was demonstrated in the study of Berrada et al. (2005). In this study, the treatments were initiated when the tumors reached a volume of approximately 100 mm^3. The C/GP-containing camptothecin was found to be more effective than the systemic delivery of camptothecin in delaying tumor growth. Although the camptothecin-treated group received a much higher dose intraperitoneally (60 mg/kg mouse) compared to the C/GP/camptothecin-treated group (24 mg/kg mouse), the latter group had eightfold smaller tumors than the initial group on day 8 of treatment. Tumors injected with blank C/GP showed no inhibition of growth, and had the same size as untreated tumors. Tumor from these two groups reached a size of four times the initial tumor volume (the end point of treatment) on day 7. Tumors from the camptothecin-treated group reached the end point on day 8, while those from C/GP/camptothecin-treated group did not reach the end point until day 25 of treatment. This strongly demonstrated the effectiveness of C/GP on the sustained release of camptothecin in vivo, and thus significantly delaying tumor growth. It also indicated that the exposure of tumor cells to drug for a prolonged period of time causes more cell death than the short drug exposure resulting from systemic administration. Furthermore, mice treated with C/GP/camptothecin showed less weight loss compared to the other groups, which implied less toxicity of the local treatment compared to systemic treatment (Berrada et al. 2005).

The effectiveness of C/GP systems were again recently investigated in the study of Han et al. (2007). Treatment was initiated on day 8 after TC-1 cervical cancer cells were subcutaneously inoculated into C57BL/6 mice. Compared to the PBS-treatment group (positive control group), C/GP hydrogel alone did not cause any inhibition of tumor growth. Using the same dose of Dox, C/GP/Dox system significantly inhibited tumor growth compared to control groups and other treatments including intravenous and intratumoral injections of free Dox. PBS-treated and C/GP alone-treated groups had tumors eight times larger compared to those at the beginning of treatment (day 8). Free Dox-treated groups had tumors of three to five times larger, while C/GP/Dox-treated groups had tumors of the same size as on day 8. This implied the greater effectiveness of a local sustained release of Dox from C/GP hydrogel matrix into the tumor site directly. It was also the result of prolonged exposure of the cancer cells to Dox as compared with a systemic administration of free Dox. In this study, vaccinia virus-based vaccine expressing Sig/E7/LAMP-1 (Vac-Sig/E7/LAMP-1) was also used as immunotherapeutic agent, which made this study as a pioneer report on the use of a biodegradable hydrogel system as an anticancer drug delivery system for successful chemoimmunotherapy. C/GP/Dox and Vac-Sig/E7/LAMP-1 were injected intratumorally and intravenously, respectively. The combination of these two treatments led to the highest tumor suppression without side effects and remarkably enhanced E7-specific $CD8^+$ T cell immune response. The combined therapy also increased long-term antitumor activity and mice survival than monotherapy alone.

21.3.1.3 Chitosan/Glyceryl Monooleate Hydrogels

The mucoadhesive pH-sensitive chitosan/glyceryl monooleate (C/GMO) in situ gel system was developed by Ganguly and Dash (2004). It employs the pH-sensitive property of chitosan solutions at low pH. Once injected into the body, these polymer solutions face different environmental pH conditions and form gels. The mucoadhesive and in situ gel-forming properties of chitosan and GMO can be used in sustaining the delivery of both hydrophilic and hydrophobic drugs, and targeting these to cells producing mucin (Larsson 1989, Dash et al. 1990, Wyatt and Dorschel 1992, Fiebrig et al. 1995, Takeuchi et al. 1996, Nielsen et al. 1998, Lee et al. 2005). An in vitro study of paclitaxel delivery from C/GMO systems and its transport across different mucin-producing cell lines demonstrated the strong potential of these in situ gels to be used as controlled and targeted drug delivery systems (Jauhari and Dash 2006). When injected close to the site of the tumor, the ionic polymer used in the formulation becomes deprotonated and will form an instant gel at the site of injection at body pH. This system can provide a sustained release of paclitaxel from the gel at and around the site of cancer, which is impossible to achieve with systemic drug administration. However, an in vivo study of C/GMO/paclitaxel in mice has not been reported as yet but is eagerly awaited.

21.3.1.4 Chitosan/Dibasic Orthophosphate Hydrogels

Recently, Ta et al. have developed a neutral and biocompatible hydrogel system based on chitosan and an inorganic orthophosphate salt (Ta et al. 2009d). In the presence of dibasic phosphate salt such as dipotassium hydrogen orthophosphate (DHO), the acidic chitosan solution was neutralized and gelling at temperature and time regulated by varying chitosan and salt concentrations in the formulation. It was found that these hydrogel systems can release entrapped agents in a sustained manner, and the release rate can be controlled by the chitosan concentration, the DHO concentration, the structural conformation, and the molecular weight of the entrapped agents. Chitosan/DHO (Chi/DHO) hydrogels have been formulated to be gelled at physiological temperature (37°C) for the delivery of Dox (Ta et al. 2009c), a common chemotherapeutic agent, and a plasmid encoding pigment epithelium-derived factor (pPEDF) (Ta et al. 2009b) in the treatment of osteosarcoma (OS). OS is a class of cancer originating from bone, mainly afflicting children or young adults (Ta et al. 2009a). It is the second-highest cause of cancer-related deaths in these age groups, mainly due to the development of often-fatal metastasis, usually in the lungs. Survival rates for these patients are poor despite the aggressive use of surgery, chemotherapy, and/or radiotherapy. Lungs are the

predominant site for OS metastasis, which in quite a number of cases is life threatening. The current management of primary OS and its secondary metastasis is limited by the lack of an efficient drug delivery system.

In situ gelling Chi/DHO hydrogel system was designed to directly deliver the frontline chemotherapeutic agent (Dox) in a sustained time period to OS sites (Ta et al. 2009c). A significant reduction of both primary and secondary OS in a clinically relevant orthotopic model was measured when Dox was administered with the hydrogel. On day 12 posttreatment, Chi/DHO–Dox reduced the tumor volumes by approximately 50% compared to untreated (control) groups. A histological analysis of tumor tissues demonstrated that Chi/DHO–Dox induced the highest level of cell apoptosis (37%), while free Dox and Chi/DPO induced less than 15% apoptosis. X-ray images revealed lesser evidence of bone degradation (osteolysis) in the groups of Chi/DHO–Dox. Histology analysis also confirmed less osteolysis in this treatment group, while significant osteolysis occurred in the other cohorts of mice. In addition to the activity of Chi/DHO–Dox at the primary bone tumor site, its activity at the secondary tumor site was also observed. The number of pulmonary (lung) metastases decreased approximately 1.5-fold in the Chi/DHO–Dox group, compared to those in the other groups. A histological examination of dissected lungs also revealed smaller metastases in the Chi/DHO–Dox group. These results indicate that Chi/DHO–Dox treatment had significantly better ability to delay metastasis growth, compared to the free Dox treatment with the same Dox dose. This hydrogel delivery system also reduced the cardiac and dermal toxicity of Dox in mice. The obtained results can be explained by the localized and sustained release of Dox from Chi/DHO hydrogel, leading to the prolonged and continuous direct actions of Dox on cancerous cells.

Currently, gene therapy is being evaluated as a novel method for OS treatment. Ta et al. have reported that the in situ gelling of Chi/DHO hydrogel system could sustain the release of a potential anticancer gene (pigment epithelium-derived factor [pPEDF]) to the tumor site (Ta et al. 2009b). A significant reduction of the primary OS in a clinically relevant orthotopic model was measured. Chi/DHO-pPEDF reduced the tumor volumes by approximately 37% compared to the control group. Interestingly, the combination of plasmid treatment and chemotherapy together with the use of this delivery system led to the highest suppression of tumor growth without side effects. The combination of Chi/DHO–Dox and Chi/DHO-pPEDF (combination treatment) mostly eliminated the primary tumors. Some mice in this treatment group did not show any tumors at the end of the study. Despite the demonstrated potent activity of Chi/DHO-pPEDF at the primary bone tumor site, the incorporation of plasmid in Chi/DPO hydrogel did not show any positive effect on the development of pulmonary (lung) metastases. However, in the combination treatment group, the number of metastases significantly reduced by approximately eightfold, indicating a synergic effect due to the dual-pronged therapy. In some mice belonging to this group, metastases were absent altogether. The results obtained from these studies demonstrate the potential application of the Chi/DHO hydrogel technology as an anticancer drug delivery system for successful chemo-gene therapy.

21.3.2 Chitosan Nanoparticles for Targeted Delivery of Therapeutics

In conventional chemotherapy, cytotoxic drugs are distributed evenly throughout the body with detrimental effects on healthy cells, causing multiple side effects such as cardio-toxicity, hair loss, or digestive problems. The ability to specifically target the drugs to cancer cells has the potential to greatly decrease the side effects by delivering high doses of the therapeutic agents only to specific sites where required. Targeted delivery has the potential to revolutionize current methods of cytotoxic drug administration to improve the clinical outcomes for cancer patients.

In principal, nanoparticle delivery of anticancer drugs to tumor tissues can be achieved by either passive or active targeting. Passive targeting takes advantage of the inherent size of nanoparticles and the unique properties of tumor vasculature, such as the enhanced permeability and retention (EPR) effect and the tumor microenvironment (Wang et al. 2008). Angiogenesis is crucial to tumor progression. Unlike those in normal tissues, angiogenic blood vessels in tumor tissues have gaps

as large as 600–800 nm between adjacent endothelial cells. This defective vascular architecture coupled with poor lymphatic drainage induces the EPR effect, allowing nanoparticles to extravasate through these gaps into extravascular spaces and accumulate inside tumor tissues. There are several chitosan nanoparticles developed for the passive targeting of therapeutics to tumors (Mitraa et al. 2001, Fukumori and Ichikawa 2006, Nam et al. 2009). However, these drug delivery systems using a binary structure conjugate inevitably have intrinsic limitations to the degree of targeting specificity they can achieve. The strategy to overcome the limitations of passive targeting is to conjugate a targeting ligand or an antibody to nanoparticles.

In actively targeted delivery technology, the surface of carriers such as nanoparticles can be functionalized with signaling molecules such as antibodies, peptides, and other small molecules that will target the carriers to the disease areas that require medication. By incorporating a targeting molecule that specifically binds an antigen or receptor that is either uniquely expressed or overexpressed on the tumor cell surface, the ligand-targeted approach is expected to selectively deliver drugs to tumor tissues with greater efficiency. Magnetic nanoparticles, composed of a magnetic (e.g., iron oxide) core and a biocompatible polymeric shell, are also effective targeted drug delivery systems. The particles encapsulate drugs and can be targeted to a desired treatment location by externally localized magnetic steering. The systemic toxicity is thus minimized and local therapeutic effects are increased. Improvement in drug dose at treatment target is also achieved.

Recently, Tan et al. synthesized chitosan nanoparticles (NPs) encapsulated with quantum dots (QDs) and used such nanomaterial to deliver human epidermal growth factor receptor-2 (HER2/neu) short-interfering RNA (siRNA) to breast cancer cells to achieve silencing effects on the HER2 gene via RNAi (Tan et al. 2007). The presence of fluorescent QDs in the chitosan NPs helped in monitoring the siRNA that is emerging as a robust method of controlling gene expression with a large number of applications. Gene silencing using siRNA is fast becoming an attractive approach to probe gene function in mammalian cells. Water-soluble green fluorescent CdSe/ZnS QDs were mixed with 0.04 w/v% chitosan solution at a chitosan:QD mass ratio of 1:1. Cationic chitosan binds to anionic water-soluble QDs readily due to the electrostatic attraction between two oppositely charged species, forming monodisperse chitosan/QD NPs around 60 nm. Chitosan NPs with encapsulated QDs that were formed as a result were purified by centrifugation and then stored in water. In order to conjugate HER2 siRNA onto the chitosan/QD NPs, HER2 siRNAs was incubated with three chitosan/QD NPs for an hour at room temperature. Unconjugated HER2 siRNA was then removed by centrifugation. Targeted delivery of HER2 siRNA to HER2-overexpressing SKBR3 breast cancer cells has been obtained by labeling the surface of chitosan/QD nanoparticles with HER2 antibody, which targets the HER2 receptors on SKBR3 cells. HER2 antibody was conjugated to the chitosan/QD NPs at 4°C overnight using established EDC/NHS chemistry.

The HER2/neu gene products are detected in breast cancers and are associated with oncogenesis (Tan et al. 2007). It is understood that the increased activity of the HER2 gene provides mitogenic signals to cells and protects them from apoptosis. As such, an effective form of breast cancer therapy can be provided by silencing the HER2 gene in cancer cells overexpressing HER2 gene products. It was found that compared to nontargeted HER2 siRNA-conjugated chitosan NPs, HER2 siRNA-conjugated chitosan NPs labeled with HER2 antibody could be specifically targeted to the HER2 receptors on the surface of SKBR3 cells that over-express HER2 receptors. On the other hand, no such binding of chitosan/QD NPs to MCF-7 cells that have a lower expression of HER2 receptors is observed. In addition, the effects of HER2 gene silencing was most pronounced with HER2 siRNA-conjugated chitosan/QD NPs labeled with HER2 antibody for both cell types. Therefore, targeted chitosan/QD NPs can be effectively used to deliver siRNA into cells with a high degree of specificity (Tan et al. 2007).

Kim et al. (2009) have employed magnetic method to target chitosan-coated magnetic NPs to carcinoma cells for magnetic hyperthermia (Kim et al. 2009). Hyperthermia has been recognized as a useful therapeutic modality for treating malignant tumors (Chan et al. 1997). There is a wide spectrum of hyperthermic applications including the use of hot water, capacitive heating,

and induction heating. However, most of these methods damage both normal and tumor cells. Therefore, intracellular hyperthermia methods have been suggested and developed using magnetic nanoparticles (Masashige 2002). Hyperthermic application requires these magnetic nanoparticles to have a high level of magnetization for high thermal energy. The delivery of hyperthermic thermoseeds to a specific target site with minimal side effects is an important challenge in targeted hyperthermia, which employs magnetic method and functional polymers. An external magnetic field is used to control the site-specific targeting of the magnetic nanoparticles. Polymer-coated magnetic nanoparticles can confer a higher affinity to the biological cell membranes.

In Kim et al.'s study, exothermic chitosan- and starch-coated magnetic nanoparticles were synthesized by controlled coprecipitation for use as a hyperthermic thermoseed (Kim et al. 2009). As in vitro tests, the magnetic responsiveness of chitosan- and starch-coated magnetite was determined by a simple blood vessel model under various intensities of magnetic field. In addition, the temperature changes under an alternating magnetic field were observed. It was found that the chitosan-coated magnetic nanoparticles generated a higher ΔT of 23°C under an AC magnetic field than the starch-coated magnetite. The capturing rate of these particles was 96% under an external magnetic field of 0.4 T. The cell-affinity test was measured in a magnetic field of 0.4 T. The cell affinities of the uncoated, chitosan-coated, and starch-coated magnetic nanoparticles were evaluated from the magnetic capturing of KB (carcinoma) and L929 (fibroblast) cells. The cell capture rate of the chitosan- or starch-coated magnetic nanoparticles had a higher value than the uncoated magnetic nanoparticles. In comparison to uncoated magnetic nanoparticles (42.1%), the chitosan-coated (73.4%) and starch-coated (64.1%) magnetic nanoparticles showed a significant ($p < 0.05$) increase in 1 day. Comparing the rate of KB cells capture with the rate of L929 cells capture, the rate of KB cells capture relatively increased with 10.8% in chitosan-coated magnetic nanoparticles. These finding indicated that chitosan-coated magnetic nanoparticles had a selective affinity to KB cells. The targeting of magnetic nanoparticles in hyperthermia was improved using a controlled magnetic field and a chitosan coating. Therefore, chitosan-coated magnetic nanoparticles are expected to be promising materials for use in magnetic targeted hyperthermia (Kim et al. 2009).

Recently, Sun et al. have developed magnetic chitosan NPs that carry and target photodynamic therapy agents to tumor sites using external magnetic fields (Sun et al. 2009). Photodynamic therapy (PDT) has become an increasingly recognized alternative to cancer treatment in the clinic. In PDT, drug action is controlled by a light source, typically a laser, transferred through fiber. In situ photosensitization of a nontoxic sensitizer generates cytotoxic reactive oxygen species (ROSs) that cause cell death and necrosis of tumor components, with minimal damage to the surrounding tissue (Dougherty et al. 1998). Selectivity is a major obstacle to be overcome by various drugs such as photosensitizers to reduce the risk of side effects. However, PDT therapy agents, namely photosensitizers (PS), are limited in application as a result of prolonged cutaneous photosensitivity, poor water solubility, and inadequate selectivity, which are encountered by numerous chemical therapies. Magnetic chitosan nanoparticles provide excellent biocompatibility, biodegradability, nontoxicity, and water solubility without compromising their magnetic targeting. More importantly, the magnetic nanoparticles have other favorable features such as MRI visibility for MRI imaging and nanoparticle tracking (Lu et al. 2007).

In this study, magnetic targeting chitosan nanoparticles (MTCNPs) were prepared and tailored as a drug delivery system and imaging agents for PS, designated as 2,7,12,18-tetramethyl-3,8-di-(1-propoxyethyl)-13,17-bis-(3-hydroxypropyl)porphyrin (PHPP) (Sun et al. 2009). Results showed that PHPP–MTCNPs could be used in MRI-monitored targeting PDT with excellent targeting ability. An in vitro experiment confirmed the intracellular uptake of PHPP–MTCNPs in human SW480 colon carcinoma cells, predicting available PDT effects as PDT damage depends on the uptake of PS by tumor cells. It was found that PHPP–MTCNPs possessed nontoxicity and excellent biocompatibility on SW480 carcinoma cells within 0–100 μM. The high photodynamic efficacy on SW480 colon carcinoma cells both in vitro and in vivo were achieved with this method. The combination of 24 h exposure of tumor cells to PHPP–MTCNPs followed by laser-irradiation-induced dose- and

light-dependent cytotoxicity on the tumor cells. In the in vivo study for photodynamic therapy, after xenografts reached 1000 mm^3 in size, PHPP–MTCNPs were administered intravenously to grafted mice followed by exposing to an externally localized magnetic field (1 T). Every 2 h, magnetic targeting was suspended and MRI signals in a mouse were monitored. When a typically low intensity *T*2 weighted image was observed in the tumor area, PDT was performed on the other xenografted mice by irradiation with 650 nm laser. Compared to the control group, the targeting PDT treatment group showed a significant tumor regression, indicating the excellent targeting ability of PHPP–MTCNPs without compromising the photodynamic efficacy. Despite the considerable depth of tumor invasion, targeting PDT mediated a substantial pathologic response. However, it was difficult for complete tumor regression. The tumor recurrence rate was 80% 14 days after first treatment. It was likely that clinically many treatment cycles would be needed to treat such comparatively large tumor masses. It was also noticeable that the localization of nanoparticles in skin and hepatic tissue was significantly less than in tumor tissue, therefore photosensitivity and hepatotoxicity can be attenuated. This particle could thus also be used to attenuate the hepatotoxicity of a large number of traditional drugs that are effective but hepatotoxic (Sun et al. 2009).

21.3.3 Cancer Detection by Chitosan Nanoparticles

Cancer is one of the major causes of mortality, and the worldwide incidence of cancer continues to increase. Early diagnosis of cancer is crucial in order to provide patients an efficient treatment. Currently, biopsy and noninvasive imaging approaches, including x-ray-based computer-assisted tomography (CT), positron emission tomography (PET), single-photon emission tomography, and magnetic resonance imaging (MRI), are used as important tools for the detection of human cancer (Wang et al. 2008). However, these conventional imaging approaches focus mainly on delineating the morphological features of the tumor, tissue, and organs, such as the anatomic location, extent, and size of the tumor, at various levels of spatial resolution and contrast. Despite continuous improvements in spatial resolution with advanced imaging equipment, imaging modalities using nontargeted contrast agents such as CT and MRI have limited sensitivity and are unable to provide specific and functional information on the disease. Tumor-targeted contrast agents based on nanoparticle formulation have been widely developed and are promising in offering enhanced sensitivity and specificity for in vivo tumor imaging using currently available clinical imaging modalities. Nanoparticles carrying contrast agents can be functionalized with signaling molecules such as antibodies and peptides, which facilitates the detection process by targeting and concentrating the contrast agents to tumor sites, thus providing more sensitive analysis.

As mentioned previously, magnetite has been investigated most widely for targeted cancer therapy and diagnosis. They have been used for magnetic field-assisted targeting of nanoparticles and were used in magnetic resonance imaging (MRI) as contrast-enhancing agents for the purpose of cancer diagnosis. Bae et al. have developed pluronic/chitosan nanocapsules encapsulating iron oxide nanoparticles (Bae et al. 2008). These nanocapsules were produced by dispersing hydrophobically modified iron oxide nanoparticles and amine-reactive pluronic derivatives in an organic solvent, and subsequent emulsification in an aqueous chitosan solution by ultrasonication. The resultant shell cross-linked nanocapsules had unique core–shell-type nanoreservoir architecture: an inner core encapsulating magnetic nanoparticles and a hydrophilic pluronic/chitosan polymer shell layer. Although no in vivo study regarding these nanoparticles has been reported yet, an in vitro study has demonstrated the efficient internalization of these nanocapsules by human lung carcinoma cells upon exposure to an external magnetic field, suggesting their promising application for the magnetically triggered delivery of various anticancer agents and contrast agents for cancer diagnosis with magnetic resonance imaging. Sun et al. have also developed magnetic chitosan nanoparticles that carry photosensitizers for cancer photodynamic therapy, and provide magnetic resonance imaging of tumor for diagnosing and monitoring the targeting photodynamic therapy (Sun et al. 2009). Results showed that these nanoparticles provided excellent targeting and imaging ability in an in

vivo study with colon carcinoma cells. Gadolinium-loaded chitosan nanoparticles were also studied for cancer treatment and diagnosis (Tokumitsu et al. 1999). Gadolinium (Gd) has been widely used as a key element of a contrast agent in MRI diagnosis. It is also a radiosensitizer used in cancer neutron capture therapy.

Photodynamic medicine is a novel approach for cancer detection and treatment via different photosensitizers and suitable light source. The 5-aminolevulinic acid (5-ALA) can be converted into protoporphyrin IX (PpIX) that can be utilized as a fluorescent probe for tumor detection. However, the fluorescent quantity of PpIX is sometimes low for detection. Tsai et al. have developed chitosan nanoparticles encapsulating 5-ALA (CNA) by ionic gelation method (Tsai et al. 2008). The loading efficiency of 5-ALA in CNA was up to 90%. They have shown that the uptake of these nanoparticles in HeLa cancer cells was greatly enhanced by ultrasound and thus increased the fluorescent quantity of PpIX. According to this concept, they have designed a novel photodynamic detection system to enhance the accuracy of diagnosis for early cervical cancer.

Semiconductor quantum dots (QDs) are nanometer-scale, light-emitting particles with unique optical and electronic properties such as size-tunable light emission, improved signal brightness, enhanced stability of the fluorescent signal, and the ability to simultaneously excite multiple fluorescent colors (Wang et al. 2008). These properties are most promising for improving the sensitivity of molecular imaging and quantitative cellular analysis by one to two orders of magnitude. QDs are excellent optical imaging nanoprobes for evaluating the specificity of tumor targeting ligands in vitro in tumor cells and in vivo in animal tumor models. Tan et al. have developed QD-based chitosan nanoparticles for the targeted delivery of siRNA to HER2-overexpressing SKBR3 breast cancer cells by labeling the nanoparticle surface with HER2 antibody (Tan et al. 2007). Using such a construct, the delivery and transfection of the siRNA can be monitored by the presence of fluorescent QDs in the chitosan NPs. Although the authors of this study did not focus on the ability of these fluorescent nanoparticles in cancer diagnosis, the results have shown a promising application of these systems in tumor imaging and detection.

Colorectal cancer is commonly diagnosed by endoscopy (Yang et al. 2008). There are two methods to improve the diagnostic sensitivity. They are chromoendoscopy and magnifying endoscopy. Color-contrast dye, such as indigo carmine, usually is sprayed on the surface of intestine and can accumulate in the pits and crevices of the mucosa to enhance the identification of the lesion. However, this color-contrast dye does not have target-specific property. Therefore, Yang et al. have conjugated chitosan chains with target-specific ligand, such as folic acid and prepared folic acid/chitosan nanoparticles (fCN) by ionic gelation method (Yang et al. 2008). fCN was then loaded with indigo carmine (fCNIC) by the same method. fCN and fCNIC were both at the range of 120–140 nm in size, 20 mV in zeta potential. The loading efficiency of indigo carmine in fCNIC was in the range of 60%–70% depending on the concentration of acetic acid and conjugation ratio of folic acid. Results showed that the adhesion of fCNIC on HT-29 colon cancer cell increased with the increase in the incubation time and in the conjugation degree of folic acid in fCNs. It suggested that fCNIC could be a promising vector of colon-specific targeting system. According to this concept, Yang et al. have been designing a novel detection system to enhance the accuracy of endoscopic diagnosis for colorectal cancer.

21.4 CONCLUSION

In recent years, chitosan has gained much interest in biomedical fields for a range of applications. The anticancer activities of chitosan have been widely investigated and applied in cancer treatment. It has also been formulated in several drug delivery hydrogel systems. Injectable chitosan hydrogels have been developed for the treatment of various cancers such as lung, breast, cervical cancer, fibrosarcoma, and mucin-production-associated cancers. The in situ gelation of these systems can be induced by UV irradiation, temperature, or pH. Drug-incorporated chitosan hydrogel solutions have been injected into the body via intratumoral administration or subcutaneous administration

next to the tumor, and have resulted in the suppression of angiogenesis and tumor growth in vivo. Although in situ forming hydrogels have not been investigated for their ability to treat distal metastases, they have shown great potential in localized cancer treatment and in preventing local tumor recurrence. Their ability in delaying the development of metastasis was also reported. Targeted chitosan nanoparticles, on the other hand, have been developed to specifically deliver therapeutics or contrast agents to tumor or metastasis site for cancer treatment and diagnosis. Theoretically, after the systemic administration of these systems into the body, they circulate in the bloodstream and specifically bind to the targeting cancerous cells or tumor sites due to the incorporation of signaling molecules in their constructs. The ligand-targeted approach is expected to selectively deliver drugs to tumor tissues with greater efficiency. It also improves the early diagnosis of cancer using current molecular imaging techniques due to the targeted delivery of contrast agents to tumor sites. This approach prolongs the presence and increases the concentration of these agents, and thus enhances the sensitivity and accuracy of tumor imaging. Although in vivo studies of targeted chitosan nanoparticles are currently limited, results from in vitro studies have demonstrated their promise for applications in cancer treatment and diagnosis.

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22 Antidiabetic Activity and Cholesterol-Lowering Effect of Chitin, Chitosan, and Their Derivatives

Chang-Suk Kong and Se-Kwon Kim

CONTENTS

22.1 INTRODUCTION

Diabetes mellitus is a highly prevalent metabolic disease, in which the pancreas does not produce enough insulin to meet its need or the body does not effectively use the insulin it produces. This leads to the elevated levels of blood glucose (hyperglycemia), which can induce the spillage of glucose into the urine (Hayashi and Ito 2002; Hayashi et al. 2006). Diabetes mellitus is classified into two major types as type 1 and type 2. Type 1 is known as insulin-dependent or childhood-onset diabetes mellitus (IDDM) and is characterized by the destruction of the insulin-producing β cells in the pancreatic islets of Langerhans, which leads to loss of insulin secretion. Type 2 is formerly called noninsulin-dependent or adult-onset diabetes mellitus (NIDDM), which is further subdivided into obese or lean (nonobese) type, and is caused by the body's ineffective use of insulin. It is often caused from excess body weight and physical inactivity and is defined by a raised fasting or postchallenge blood glucose level (Do et al. 2008). Diabetes in clinical diagnosis usually accompanies the symptom of hypercholesterolemia with hyperglycemia, which can damage blood vessels, referred to as microvascular disease, and increases the risk of heart attack, stroke, and kidney failure (Dieterle et al. 2006). Therefore, hyperglycemia and hypercholesterolemia are well-known major cardiovascular risk factors in type 2 diabetes. The hyperglycemic and hypercholesterolemic activities in diabetic animal models have been adopted for antidiabetic study. Streptozotocin (STZ)-induced diabetes has been well recognized in animal studies for type 1 and type 2 diabetes. STZ is particularly toxic to the insulin-producing beta cells of the pancreas in mammals.

For the past few decades, a number of researches have focused on chitin, chitosan, and their derivatives as a source of potential bioactive material. Chitosan and chitin consist of 2-amino-2-deoxy-(1–4)-β-D-glucopyranose residues (D-glucosamine units) and 2-acetamido-2-deoxy-(1–4)-β-D-glucopyranose

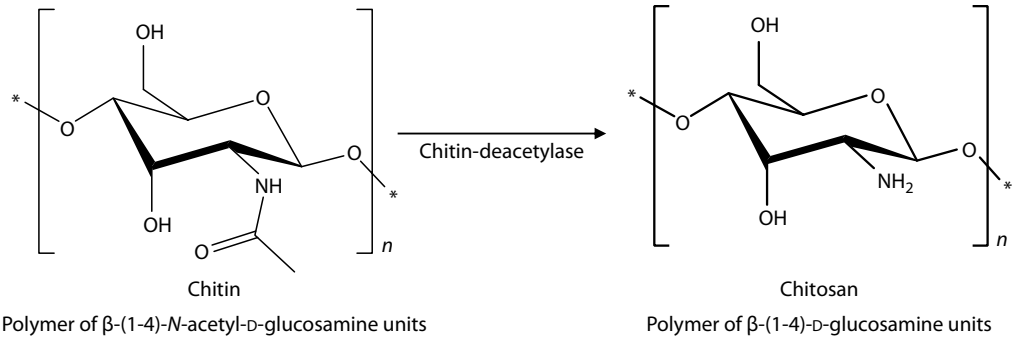

FIGURE 22.1 Structure of chitin and chitosan.

residues (*N*-acetyl-D-glucosamine units), respectively (Je and Kim 2005) (Figure 22.1). Chitosan is derived commercially by an alkaline deacetylation of chitin. Chitosan and chitin have been used considerably for their commercial applications in biomedical, food, and chemical industries (Muzzarelli 1997). The chemical modification of chitosan and chitin has been conducted to overcome the lower solubility due to high molecular weight and high viscosity. In fact, a wealth of evidence on the various biological activities of chitin, chitosan, and their derivatives has been reported (Senel and McClure 2004; Kanatt et al. 2008; Laurencin et al. 2008). In this chapter, we describe the antidiabetic activity and the cholesterol-lowering effect of chitin, chitosan, and their derivatives.

22.2 ANTIDIABETIC ACTIVITY

The antidiabetic effects of chitosan and its derivatives were reported in diabetic animal models, type 1 and type 2. A number of studies have been reported in the literature.

22.2.1 Chitosan

The antidiabetic effect of chitosan has been reported in two types of diabetic animal models, type 1 and type 2. At first, Miura et al. (1995) reported that chitosan given as a 5% food mixture produces consistent blood glucose- and lipid-lowering effects in normal mice and neonatal STZ-induced diabetic mice (one of the animal models of nonobese type NIDDM). Low molecular weight chitosan (LMW: 20,000 Da) prevents the progression of low-dose STZ-induced slowly progressive diabetes mellitus in male ICR mice (Ito et al. 2000). The daily administration of LMW chitosan as drinking water prevents the progression of low-dose STZ-induced slowly progressive NIDDM in mice (Kondo et al. 2000). This progressive diabetic model is nonobese NIDDM, which is characterized by an impaired insulin response to glucose stimulation (Ito et al. 2001). Hayashi and Ito (2002) designed to clarify the effects of LMW chitosan on hyperglycemia, hyperinsulinemia, and hypertriglyceridemia in genetically obese diabetic male KK-A^y mice, one of the animal models of genetically obese type NIDDM with hyperinsulinemia. LMW chitosan lowered the serum glucose levels and improved overdrinking and polyuria observed in these diabetic mice. It is concluded that LMW chitosan is useful for the treatment of obesity-related type 2 diabetes mellitus.

The antidiabetic effects of high molecular weight (HMW: 200,000–300,000 kDa) chitosan on STZ-induced type 1 diabetic ICR mice were investigated by Do et al. (2008). The antidiabetic effects were tested by analyzing food consumption, body weight, drinking water consumption, urine volume, nonfasting serum glucose, urine glucose, total serum cholesterol, and triglyceride levels. HMW chitosan (0.8%) was given to mice as drinking water for 8 weeks. Daily administration of HMW chitosan reduced the consumption of drinking water, urine volume, nonfasting serum glucose, urine glucose, and serum triglyceride levels. It is concluded that HMW chitosan would be useful for relieving type 1 diabetes mellitus.

Yao et al. (2008) compared the antidiabetic activities of high and low molecular weight chitosan by evaluating the hypoglycemic and hypocholesterolemic effects in STZ-induced diabetic rats. Rats were divided into three groups of normal rats, a cellulose (control) diet, LMW (14,000 Da) chitosan diet and HMW (1,000 kDa) chitosan diet groups. HMW or LMW chitosan diets had increased high density lipoprotein (HDL) cholesterol. Although chitosan did not affect plasma glucose in normal rats, HMW chitosan diet group significantly decreased plasma glucose and total cholesterol, and increased HDL cholesterol and fecal cholesterol excretion compared to the cellulose diet and LMW chitosan diet groups. This study demonstrated the potential of HMW chitosan in reducing hyperglycemia and hypercholesterolemia in STZ-induced diabetic rats.

The application of chitosan as a vector in gene therapy for type 1 diabetes mellitus was studied for chitosan nanoparticles (Niu et al. 2008). This study revealed that the human insulin gene can be transfected successfully by chitosan nanoparticles in vitro and in vivo. This study supported the application of chitosan in the gene therapy of diabetes in living organisms as a safe vector without causing any pain.

22.2.2 Chitosan Derivatives

Chitosan derivatives including chitooligosaccharides (COS) possess various biological activities and can be used in the treatment of diabetes mellitus. The antidiabetic effect of chitosan derivatives has been developed by the water soluble COS (Lee et al. 2003; Liu et al. 2007). The antidiabetic effects of COS were tested in neonatal STZ-induced NIDDM rats, and its proliferative effect were tested on pancreatic islet cells in STZ-induced diabetic rats. Lee et al. (2003) described that COS has a TG-lowering effect in diabetic rats, and COS reduces the signs of diabetic cardiomyopathy such as the vacuolation of mitochondria and the separation and degeneration of myofibrils. These results conclude that COS can be used as an antidiabetic agent because it increases glucose tolerance and insulin secretion and decreases TG. Liu et al. (2007) prepared the soluble COS with low molecular weight by the enzymatic hydrolysis of chitosan and investigated the proliferative and antidiabetic effects of COS on pancreatic islet cells in STZ-induced diabetic rats. This study described that COS could effectively accelerate the proliferation and insulin secretion of pancreatic islet cells. Moreover, COS could improve the general clinical symptoms of diabetic rats, decrease the 2 h plasma glucose and urine glucose, and normalize the disorders of glucose tolerance.

Recently, new basal insulin formulation was designed by Jo et al. (2008). Zinc-crystallized insulin was physically loaded into hydrophobically modified glycol chitosan (HGC) nanoparticles by dialysis method (Figure 22.2). The biological activities of insulin–HGC formulations were investigated

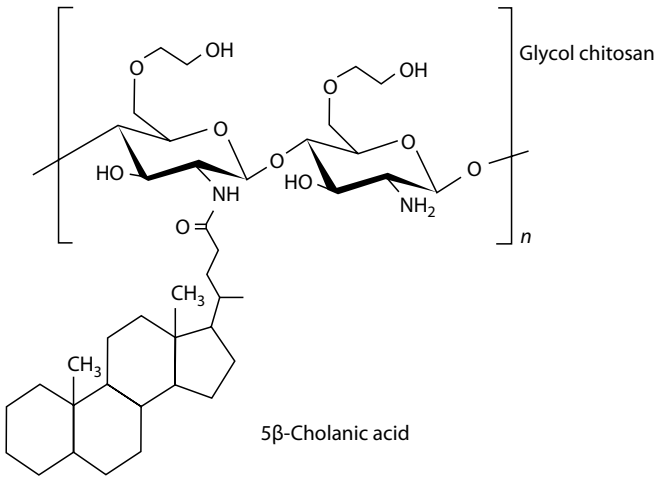

FIGURE 22.2 Chemical structure of HGC conjugate. (From Jo, H. G., et al., *Archives of Pharmacal Research*, 31, 918, 2008. With permission.)

by examining the impact of insulin loading on the size distribution of nanoparticles, where insulin release in vitro, and its in vivo glucose-lowering effect in type 1 diabetic rats. Insulin–HGC effectively sustained insulin release up to 40% within 12 h followed by a slower controlled release. Insulin–HGC showed an extended blood glucose–lowering effect up to 24 h and provided normal blood glucose levels after oral glucose (1.5 g/kg) load at 24 h postinjection, although regular insulin exhibited severe hypoglycemia. The prolonged time action profiles and low variability of insulin–HGC formulation induced the improved blood glucose control in diabetic rats and fulfilled a pattern desirable of a basal insulin. Consequently, the insulin–HGC demonstrated longer duration of action in vivo than the native form, resulted in enhanced biological potencies as a novel prolonged antidiabetic agent.

22.3 CHOLESTEROL-LOWERING EFFECT

Hypercholesterolemia is an important risk factor for cardiovascular disease. The hypocholesterolemic action of chitosan can be explained to be due to the decrease in cholesterol absorption and interference with bile acid absorption, a mechanism similar to those of dietary fiber constituents. Here, the cholesterol-lowering effects of chitin, chitosan, and their derivates are reported based on animal and clinical studies.

22.3.1 Chitin and Chitosan

A number of studies have revealed that chitin and chitosan are effective cholesterol-lowering supplements and fat blockers. When chitosan is used as a food supplement, chitosan can effectively reduce low-density lipoprotein (LDL), cholesterol, and plasma cholesterol while improves the HDL cholesterol/total cholesterol ratio (Koide 1998). These studies indicated that increased bile acid excretion and/or decreased cholesterol absorption was responsible for it (Marlett 1997).

Bokura and Kobayashi (2003) suggested that in the digestive system, chitosan acts by forming gels in the intestinal tract, which entrap cholesterol and lipids, and ingested chitosan develops an HCl layer in the stomach. As capsulated particles of chitosan move into the duodenum, the HCl layer becomes diluted, and the chitosan particles form agglomerates with cholesterol and fatty acids, thereby reducing cholesterol absorption from the gastrointestinal tract. Studies in rats demonstrated that chitosan can increase the amount of fat that is eliminated in the stool and decrease cholesterol-reducing activity (Sugano et al. 1980; Ebihara and Schneeman 1989; Gallaher et al. 2000; Tapola et al. 2008). In a randomized, double-blind, and placebo-controlled clinical study, oral chitosan reduced serum total cholesterol, especially in elderly women, while chitosan exhibited the mild effect of reducing total and LDL cholesterol (Bokura and Kobayashi 2003).

As an attempt to elucidate the interaction mechanism between chitosan and cholesterol, Pavinatto et al. (2005) probed the interaction between chitosan and cholesterol in Langmuir monolayers (Figure 22.3). Chitosan incorporated in the aqueous subphase is found to affect the Langmuir monolayers of cholesterol, causing surface pressure and surface potential isotherms to become more expanded. The interaction occurs via H-bonding between the hydroxyl groups of cholesterol and hydroxyl and amine groups of chitosan.

Tai et al. (2000) suggested a possible mechanism as follows. Dietary lipids or fats and bile are major sources of cholesterol in the intestinal lumen and contain negatively charged surfaces of phospholipids and unesterified cholesterol. Chitosan has high positive charge densities in acidic solutions. Therefore, chitosan is positively charged in gastric acid, and the positively charged chitosan interacts with negative surfaces such as cholesterol, bile acids, and lipids. The chitosan–cholesterol complex is transferred to the intestines under an alkaline condition and changes into an insoluble gel form. It cannot be hydrolyzed by pancreatic or intestinal enzymes (Kanauchi et al. 1995) and

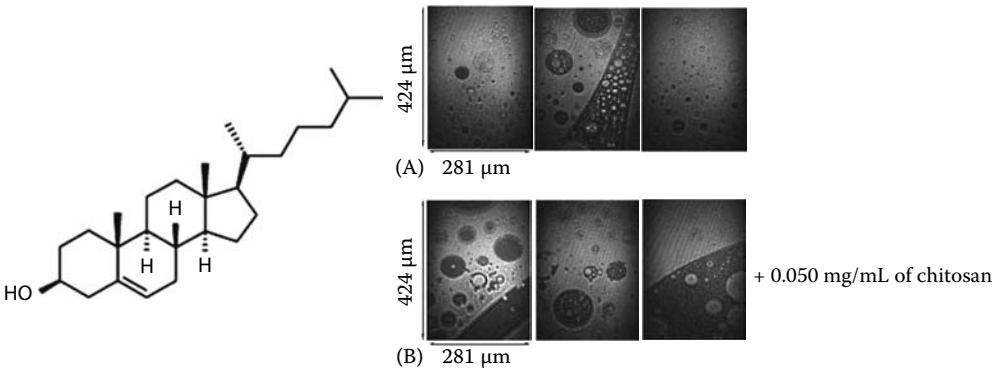

FIGURE 22.3 Chemical structure of cholesterol and Brewster angle microscopy image for cholesterol monolayers. (From Pavinatto, F. J., et al., *Polímeros: Ciência e Tecnologia*, 15, 91, 2005. With permission.)

is excreted in the feces, thereby interrupting enterohepatic circulation of the cholesterol and accelerating its excretion in stools. The effects of chitosan on fecal excretion of cholesterol and bile acid have been reported in animal and human studies or animals and humans (Lengsfeld et al. 1999; Imonek and Bartonova 2005; Yao and Chiang 2006). These studies provide that the cholesterol-lowering effect of chitosan may be primarily associated with an increase in fecal excretion of cholesterol and bile acid. As the absorption of cholesterol is decreased, consequently, the hepatic bile acid pool is depleted, the more hepatic cholesterol is diverted into the production of bile acids. Since hepatic cholesterol demands increase, hepatic cholesterol synthesis increases and LDL receptor apolipoprotein (apo)B/E activity is stimulated. Then, the stimulation of LDL receptor apoB/E activity increases the catabolism of LDL cholesterol and reduces plasma LDL cholesterol levels (Bilheimer 1988).

Xu et al. (2007) investigated the effect of chitosan on lipid metabolism in hyperlipidemic rats. Chitosan lowered total cholesterol levels, LDL cholesterol in plasma, and total cholesterol and total triglyceride in liver, and increased fecal bile acids excretion, but did not change the level of triglyceride and HDL cholesterol in plasma. In addition, chitosan increased hepatic LDL receptor mRNA levels. This study suggested that chitosan improves lipid metabolism by regulating total cholesterol and LDL cholesterol by the upregulation of hepatic LDL receptor mRNA expression, increasing the excretion of fecal bile acids.

Chitosan acts as a weak anion exchange resin and exhibits a substantial viscosity in vitro. Sugano et al. (1988) reported that changes in viscosity did not affect the hypocholesterolemic effect of chitosan, leading all to be equivalent hypocholesterolemic effects. However, Chiang et al. (2000) reported that the higher viscosity showed the greater cholesterol-lowering effect of chitosan. The anion exchange property of chitosan would seem to be favored as an explanation for its hypocholesterolemic properties. A mixture of chitosan and glucomannan decreased cholesterol absorption and increased bile acid excretion in rats fed with a cellulose-based diet (Gallaher et al. 2000). Increased bile acid excretion could reduce cholesterol concentrations because plasma or liver cholesterol would be utilized to maintain the bile acid pool.

Although the cholesterol-lowering action of oral chitosan was reported by many researchers, the cholesterol-lowering action of chitin was not clear in spite of exhibiting higher excretion of triglycerides in feces (Sugano et al. 1988; Harish Prashanth and Tharanathan 2007). Zacour et al. (1992) reported on the effect of chitin at the level of 5% in the diet on cholesterol absorption and metabolism in Wistar rats fed on diet containing beef tallow (7%) and cholesterol (1%). Chitin exhibited similar weight gain and feed efficiency, equivalent fatty liver (steatosis), lower apparent protein digestibility, decreased levels of liver triglycerides and cholesterol, similar

levels of serum and fecal cholesterol, and higher excretion of triglycerides in feces, compared with pair-fed controls.

22.3.2 Chitosan Derivatives

The cholesterol-lowering effect of COS was investigated by measuring the level of serum lipids, antioxidant enzyme activities, and lipid peroxidation in rats fed with high cholesterol diet for 4 weeks (Kim et al. 2005). COS-supplemented groups significantly decreased the serum total cholesterol, LDL cholesterol, and triglyceride levels and increased the relative HDL cholesterol level in total cholesterol. These results suggest that the supplement of chitosan oligosaccharides reduce the levels of serum cholesterol in rats fed with high cholesterol.

The binding capacity for cholesterol of *N*-alkyl chitosans was reported by Chandler and Curatolo (1992). The newly synthesized and fully characterized *N*-lauryl chitosan and *N*-dimethylaminopropyl chitosan endowed with higher hydrophobic and cationic properties to alter the compositions of olive oil (Muzzarelli et al. 2000). Tong et al. (2005) described the regioselective synthesis of an amphiphilic chitosan derivative, *O*,*O*′-dipalmitoyl chitosan, by the selective protection of amino groups in $MeSO_3H$ (Figure 22.4) and also investigated its amphiphilic behavior and miscibility with its capability of cholesterol absorption. This amphiphilic chitosan with free amino groups was cross-linked with glutaraldehyde. This cross-linked amphiphilic chitosan could effectively adsorb cholesterol both in polar and nonpolar solvents.

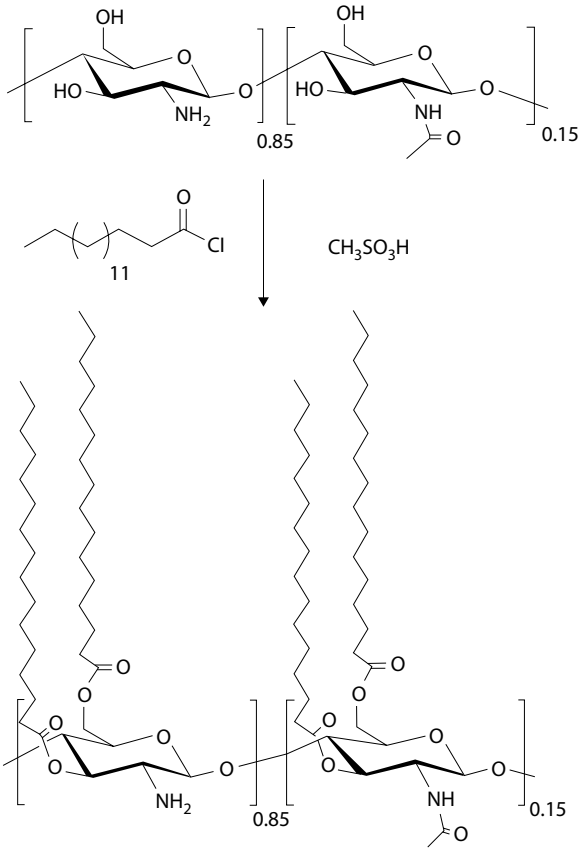

FIGURE 22.4 Synthesis of *O*,*O*′-chitosan. (From Tong, Y., et al., *Carbohydrate Polymers*, 60, 229, 2005. With permission.)

22.4 CONCLUSIONS

Chitosan and its derivative exhibited the great antihyperglycemic, antihyperlipidemic, and antihypocholesterolemic activities in type 1 and type 2 diabetic animal models. Moreover, chitin, chitosan, and their derivatives exhibited cholesterol-lowering activity in animal and clinical studies. Therefore, chitin, chitosan, and their derivatives can be promising candidates as potential material for protecting diabetes mellitus and lowering the cholesterol absorption.

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Part V

Biomedical Applications of Chitin and Chitosan Derivatives

23 Chitin/Chitosan Oligosaccharides: Effective Substrates for Functional Analysis of Chitinases/Chitosanases

Takayuki Ohnuma and Tamo Fukamizo

CONTENTS

23.1 INTRODUCTION

Oligosaccharide fragments derived from chitin and chitosan exhibit various biological activities, as described in Parts IV and VII. These useful oligosaccharides have been obtained by acid or enzymatic hydrolysis of chitin and chitosan followed by chromatographic separation. Since more controlled hydrolysis of the polysaccharide was found to be possible using chitin/chitosan degrading enzymes (so-called chitinases and chitosanases), the enzymes have been used for efficient oligosaccharide production (Aiba 1994, Lopatin et al. 1995, Cheng and Li 2000, Yoon et al. 2002, Woo and Park 2003, Choi et al. 2004). The enzymes have also attracted public attention because of their capabilities in biological control. Plant chitinases are implicated in host defense against fungal pathogens (Kasprzewska 2003), and insect chitinases are possible targets for pest control

(Kramer and Muthukrishnan 1997). Since the fungal pathogens that cause a number of human and animal diseases use chitin and chitinases in their life cycles, the fungal chitinases are possible inhibitory targets for preventive or therapeutic agents. In this situation, various kinds of chitinolytic enzymes have been isolated from living organisms, and their genes were cloned, sequenced, and expressed by a microbial expression system to produce recombinant enzymes. In characterizing the recombinant enzymes, polymeric substrates are frequently used regardless of their insolubility and heterogeneity with respect to the degree of polymerization. The results obtained from such polymeric substrates are often ambiguous and do not allow us to discuss the data quantitatively. Thus, oligosaccharide substrates are useful for the quantitative determination of the enzymatic reaction because of their high solubility and homogeneity.

Various strategies for enzymatic analysis have brought about recent progress in the understanding of the mechanisms of chitinases/chitosanases. Especially, x-ray crystallography and protein engineering techniques have significantly contributed to such a progress in enzyme research. The database (CAZy; http://www.cazy.org/) constructed by Henrissat and his coworkers has also contributed to the progress by facilitating prediction of the molecular function of these enzymes based on their amino acid sequences. As well as these strategies, contribution of various experiments using oligosaccharide substrates has been significant for obtaining important information on the enzyme structure and function. In this chapter, the authors review the recent advances in enzymatic analysis of chitinases/chitosanases using chitin/chitosan oligosaccharides.

23.2 CHITIN OLIGOSACCHARIDES

23.2.1 Preparation

Chitin oligosaccharides [$(GlcNAc)_n$, $n = 1, 2, 3, \ldots$; Figure 23.1] can be prepared by acid hydrolysis of chitin (Rupley 1964, Berkeley et al. 1972). Powdered chitin was suspended in concentrated hydrochloric acid and incubated in a bath at 40°C for 2–3 h with continuous mechanical stirring. After neutralizing the acid hydrolysate, the supernatant is applied onto a charcoal–celite column followed by linear gradient elution of ethanol (from 0% to 60%) to separate the chitin oligosaccharides from each other. This method is not always convenient especially for obtaining longer chain length oligosaccharides, such as $(GlcNAc)_5$ and $(GlcNAc)_6$. This is because the concentrated acid intensively hydrolyzes chitin-producing shorter oligosaccharides including GlcNAc monomer. The limited hydrolysis of chitin has been performed using chitinase enzyme, and the production of chitin trisaccharide was enhanced by enzymatic chitin hydrolysis (Woo and Park 2003). Longer chain length chitin oligosaccharides were obtained by chitinase hydrolysis of partially *N*-acetylated chitosan followed by *N*-acetylation (Aiba 1994, Lopatin et al. 1995). For preparative purposes, a gel-filtration column of BioGel P-2 or P-4 (BioRad Lab.)

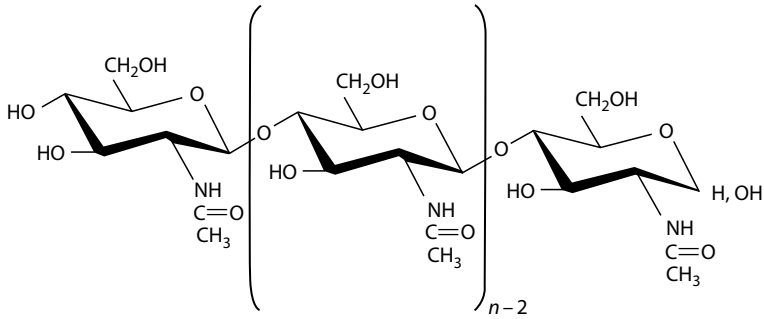

FIGURE 23.1 Chemical structure of chitin, β-1,4-linked 2-acetamido-2-deoxy-D-glucopyranose polysaccharide.

was also used for the separation of chitin oligosaccharides. Recently, a gel-filtration resin, Cellulofine GCL-25-m (Seikagaku Kogyo Co.), was found to be efficient for the purification of chitin oligosaccharides.

A more convenient production method for the longer chain length oligosaccharides was reported by Usui et al. (1990). They successfully synthesized longer chain length chitin oligosaccharides from $(GlcNAc)_2$ with the aid of a transglycosylation reaction catalyzed by hen egg white lysozyme. Ammonium sulfate was found to be useful for enhancing lysozyme transglycosylation. Gram-scale enzymatic synthesis of chitin oligosaccharides in *Escherichia coli* was achieved by Samain et al. (1997). High cell density cultivation of *E. coli* cells harboring the *nodC* gene, which encodes *Mesorhizobium loti* chitin oligosaccharide synthase, produces 2.5 g chitopentaose per liter of culture medium. This method is attractive in terms of high yields, time and cost effectiveness, reproducibility, and simple operation. It is possible to produce chitin oligosaccharide derivatives or more complex oligosaccharides in this system by coexpressing appropriate enzymes in the cell.

23.2.2 Monitoring the Chitinase Reaction toward Chitin Oligosaccharides

For analytical purposes, chitin oligosaccharides have been separated by thin layer chromatography (TLC) (Powning and Irzykiewicz 1967). A solvent system consisting of *n*-butanol:methanol:28% ammonia:water (10:8:4:2) has been employed for the separation of chitin oligosaccharides using a silica gel plate (Silica gel 60, Merck). Oligosaccharides were visualized by spraying the plate with aniline–diphenylamine reagent (4 mL of aniline, 4 g of diphenylamine, 200 mL of acetone, and 30 mL of 85% phosphoric acid) and baking them at 180°C for 3 min. The TLC method has achieved only partial success in establishing the enzyme mechanism. Hence, it is less quantitative, and the data could not be used for kinetic analysis. High-performance liquid chromatography (HPLC) should be used for obtaining fully quantitative data for kinetic analysis. HPLC determination of chitin oligosaccharides was first reported in 1977 by Eikeren and MacLaughin. They used radiolabeled oligosaccharides for enhancing the sensitivity of the assay. A similar HPLC method was used to analyze the mechanism of lysozyme hydrolysis and transglycosylation (Masaki et al. 1981a). In this report, the intact chitin oligosaccharides were detected by ultraviolet absorption at 210 nm originating from their *N*-acetyl groups, which is now generally used for the HPLC monitoring of chitin oligomers. The HPLC techniques have played important roles in the recent progress in understanding the structure–function relationship of chitinases.

A gel-filtration or partition column has been used for HPLC separation of chitin oligosaccharides. As shown in the top of Figure 23.2, oligosaccharides from GlcNAc to $(GlcNAc)_5$ were well separated by the gel-filtration column of TSK-GEL G2000PW (Tosoh), although the separation of $(GlcNAc)_5$ and $(GlcNAc)_6$ was not complete. The elution was conducted with distilled water or buffered aqueous solution. The oligosaccharides were detected by ultraviolet absorption at 220 nm, and the data obtained were fully quantitative. The separation requires a relatively long period (about 2 h) because the flow rate of the gel-filtration HPLC must be suppressed (0.3 mL/min). The hydrolysis of hexasaccharide substrate by class V chitinase from cycad (*Cycas revoluta*) was monitored by this HPLC system (Figure 23.2), and the time course of the reaction was obtained from the HPLC profiles as shown in Figure 23.3. $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$ were the major hydrolytic products, indicating that $(GlcNAc)_6$ was hydrolyzed in an endo-splitting fashion. In addition, production of oligosaccharides with polymerization degrees higher than that of the initial substrate (designated by the arrows in Figure 23.2) indicates the catalysis of transglycosylation reaction in addition to hydrolysis.

For oligosaccharides from GlcNAc to $(GlcNAc)_6$, however, more speedy separation is possible with a partition column of TSK-GEL NH_2-60 (Tosoh). The separation was completed within 15 min, when the elution was carried out with 60% acetonitrile, as shown in Figure 23.4. Ultraviolet absorption at 220 nm was also used for the oligosaccharide detection. $(GlcNAc)_5$ and $(GlcNAc)_6$ were completely separated by this system. However, the data obtained from a partition

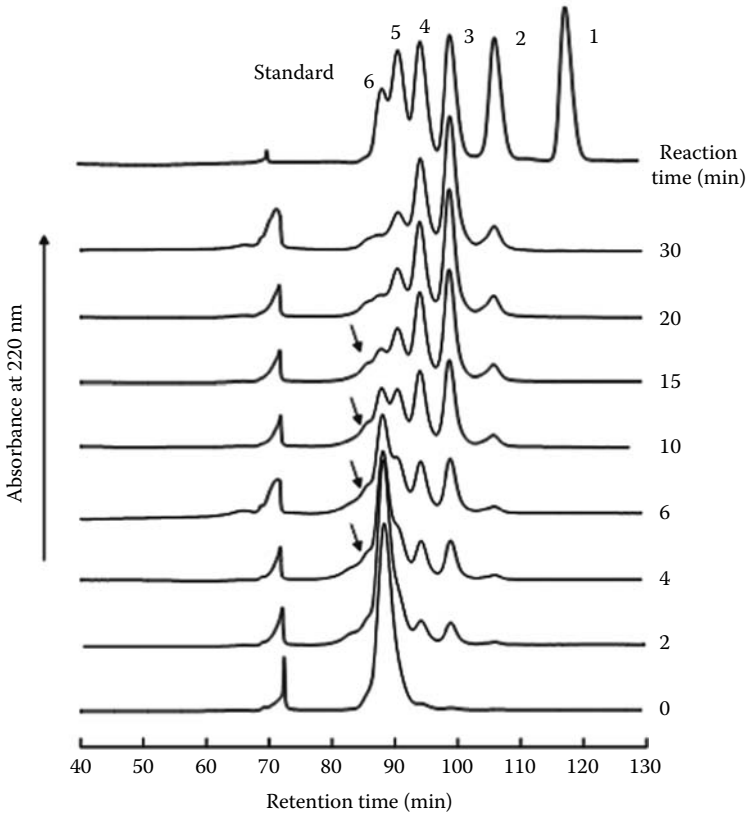

FIGURE 23.2 Time-dependent HPLC profiles showing the hydrolysis of $(GlcNAc)_6$ catalyzed by class V chitinase from cycad (*Cycas revoluta*). Enzymatic reaction was conducted in 20 mM sodium acetate buffer pH 5.0. Concentrations of the enzyme and the substrate were 0.2 μM and 4.6 mM, respectively. A gel-filtration column of TSK-GEL G2000PW was used for the separation, and the elution was conducted with distilled water and at a flow rate of 0.3 mL/min. The numerals in the chromatogram indicate the polymerization degree of $(GlcNAc)_n$. The arrows indicate the transglycosylation products.

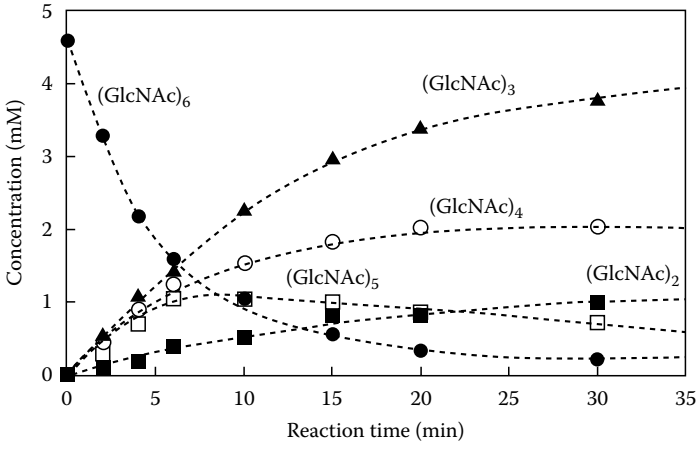

FIGURE 23.3 Time course of $(GlcNAc)_6$ hydrolysis catalyzed by class V chitinase from cycad (*Cycas revoluta*). The data points were calculated from the individual peak areas obtained by the HPLC chromatogram shown in Figure 23.2. ■, $(GlcNAc)_2$; ▲, $(GlcNAc)_3$; ○, $(GlcNAc)_4$; □, $(GlcNAc)_5$; ●, $(GlcNAc)_6$.

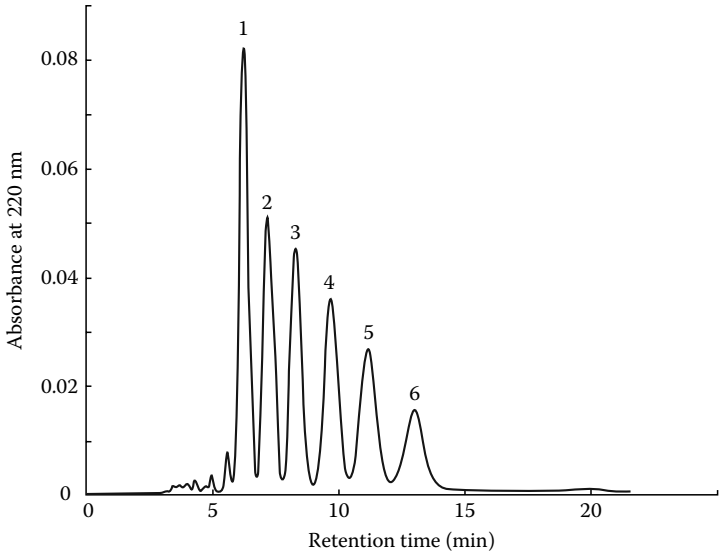

FIGURE 23.4 HPLC separation of chitin oligosaccharide mixture. Elution was performed with 60% acetonitrile and at a flow rate of 0.8 mL/min. The numerals in the chromatogram indicate the polymerization degree of $(GlcNAc)_n$.

column are generally less quantitative than those obtained by a gel-filtration column. In addition, a partition column itself is not so durable. On the other hand, using 70% acetonitrile as the elution solvent, α- and β-anomers of individual oligosaccharides were completely separated by a HPLC system equipped with a partition column of TSK-GEL Amide 80 (Tosoh) (Fukamizo and Hayashi 1982, Koga et al. 1998), as shown in Figures 23.5 and 23.6. The separation of anomeric forms affords an excellent opportunity for enzyme characterization. Monitoring the anomer formations from the oligosaccharide hydrolysis enables us to gain an insight into the catalytic mechanisms and modes of action of chitinases (Yanase et al. 1987, Koga et al. 1998). As shown in Figure 23.5, the rice class III chitinase (family GH18) predominantly produce the β-anomer of $(GlcNAc)_4$ from $(GlcNAc)_6$ (Sasaki et al. 2002). Since the α/β ratio is about 1.6 at equilibrium, the predominant formation of β-anomer indicates that the enzyme specifically produces β-anomer through the retaining mechanism in its catalytic reaction. This result also indicates that the enzyme splits the second β-1,4-glycosidic linkage from the reducing end of $(GlcNAc)_6$. Since the decrease in β-anomer of $(GlcNAc)_6$ was faster than that in the α-anomer, β-anomer of $(GlcNAc)_6$ appears to be preferentially hydrolyzed by the enzyme. On the other hand, the rice class I chitinase (family GH19) predominantly produces the α-anomer of $(GlcNAc)_2$ and $(GlcNAc)_4$, as shown in Figure 23.6. The chitinase was found to catalyze the hydrolysis of β-1,4-glycosidic linkage through the inverting mechanism (Sasaki et al. 2003). Further examination of the HPLC profiles suggested the symmetrical cleavage of $(GlcNAc)_6$ and preferential consumption of the β-anomer substrate. Based on the anomeric form of the hydrolytic products, it is now possible to classify chitinases into family GH-18 or GH-19 (CAZy; http://www.cazy.org/) and to specify their mode of action.

The enzymatic reaction of plant chitinases was investigated by electrospray ionization-mass spectrometry (ESI-MS). Hexasaccharide hydrolysis by barley class II chitinase (family GH19) was monitored in real-time by ESI-MS, producing a reaction time course that was completely consistent with that previously obtained by HPLC (Honda and Fukamizo 1998), as shown in Figure 23.7. Kinetic modeling of enzymatic hydrolysis successfully simulated the profile of the time course obtained by ESI-MS (data not shown), indicating that the reaction time course obtained by ESI-MS in real-time has sufficient accuracy (Fukamizo et al. 2008). In the ESI-MS method, the amounts

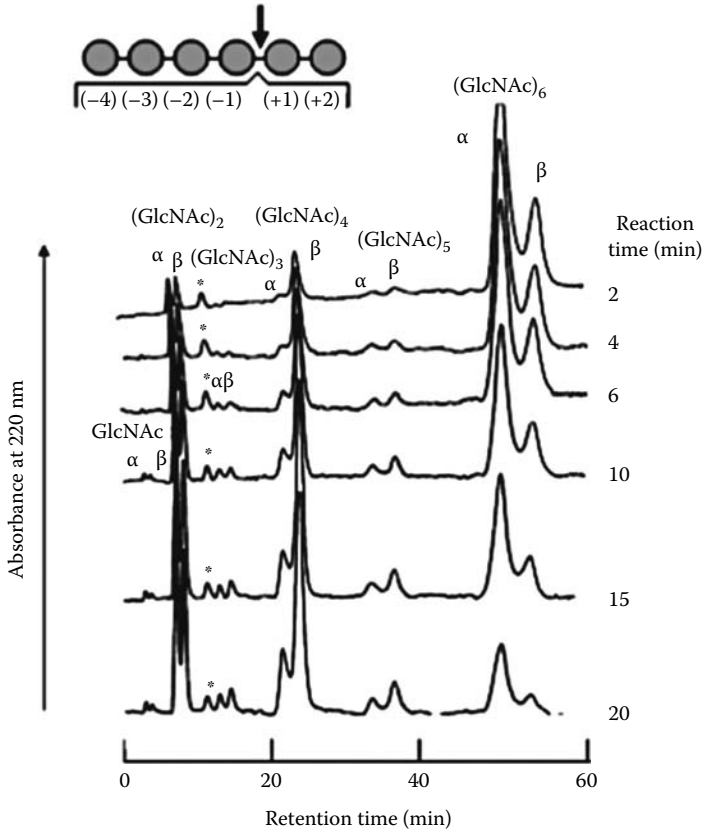

FIGURE 23.5 Anomer formation catalyzed by rice class III chitinase (family GH18) from the hexasaccharide substrate. Enzyme and substrate concentrations were 0.3 µM and 4.9 mM, respectively. The enzymatic reaction was conducted in 50 mM sodium acetate buffer pH 5.0 at 25°C. A partition column of TSK-GEL Amide 80 (Tosoh) was used with a flow rate of 0.7 mL/min. The peaks designated by * are derived from impunity.

of enzyme and substrate used were much lower than those used in the HPLC method, and the real-time monitoring achieved the much quicker determination compared with the discontinuous HPLC determination.

No significant progress of the hydrolytic reaction was observed by real-time ESI-MS of E67Q, in which Glu67 is mutated to glutamine. As previously reported (Andersen et al. 1997), Glu67 was confirmed to be the catalytic residue of the chitinase. To characterize the class V chitinase (Family GH18) from cycad (*Cycas revoluta*), we determined the reaction time course of $(GlcNAc)_6$ hydrolysis by ESI-MS in real-time (Figure 23.8). The time course obtained by real-time ESI-MS exhibited a simple hydrolytic profile, which is not consistent with the profile obtained by HPLC (Figure 23.3). In the time course obtained by HPLC, $(GlcNAc)_5$ was produced in significant amounts in addition to the products, trimer, tetramer, and dimer. $(GlcNAc)_5$ was not directly produced from the substrate $(GlcNAc)_6$, because no monomer was detected as the product. The abnormal $(GlcNAc)_5$ production indicates that the pentamer is produced through the transglycosylation reaction catalyzed by the enzyme. However, no $(GlcNAc)_5$ was detected in the time course possible through ESI-MS (Figure 23.8), indicating that the transglycosylation reaction is missing in this case. From the parallel determinations of reaction time courses by HPLC and real-time ESI-MS, we found that the transglycosylation reaction hardly takes place under the reaction conditions for real-time ESI-MS. The higher substrate concentration (4.5 mM) used for the HPLC determination is likely to enhance the transglycosylation reaction.

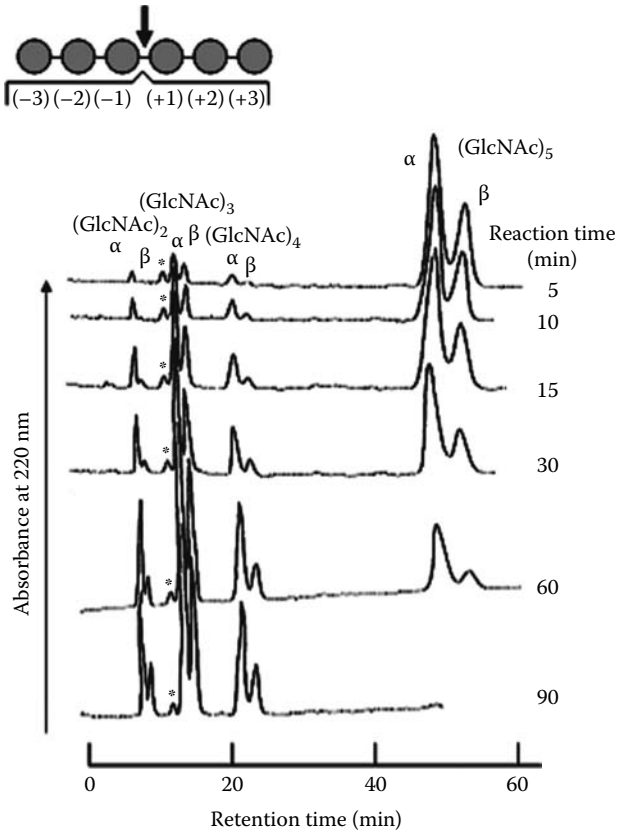

FIGURE 23.6 Anomer formation catalyzed by rice class I chitinase (family GH19) from the hexasaccharide substrate. Enzyme and substrate concentrations were 2.1 μM and 2.7 mM, respectively. The enzymatic reaction was conducted in 50 mM sodium acetate buffer pH 5.0 at 25°C. HPLC conditions were the same as in Figure 23.5.

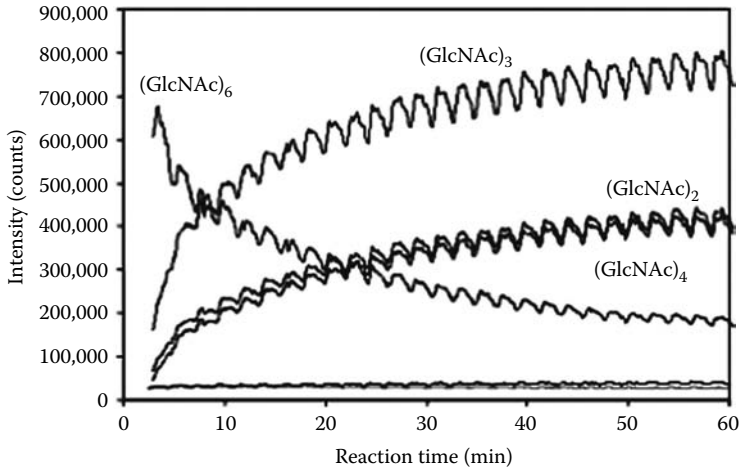

FIGURE 23.7 Real-time ESI-MS monitoring of $(GlcNAc)_6$ hydrolysis catalyzed by barley class II chitinase (family GH19). The enzymatic reactions were carried out in 10 mM ammonium acetate-containing aqueous solutions pH 5.2 at 20°C. Concentrations of the enzyme and the substrate were 0.5 and 25 μM, respectively. Time-course data were corrected with ionization factors.

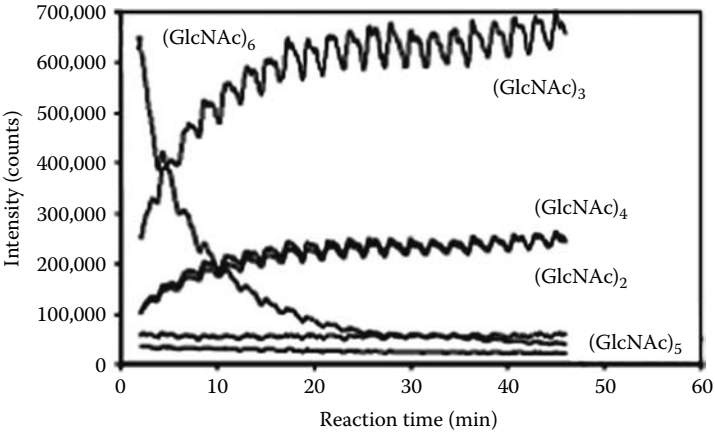

FIGURE 23.8 Real-time ESI-MS monitoring of $(GlcNAc)_6$ hydrolysis catalyzed by class V chitinase from cycad (*Cycas revoluta*). Enzymatic reaction was conducted in 20 mM ammonium acetate buffer pH 5.0. Concentrations of the enzyme and the substrate were 0.1 and 25 μM, respectively. Time-course data were corrected with ionization factors.

23.2.3 Chitin Oligosaccharide Binding to Chitinases

Fluorescence spectroscopy was used to determine oligosaccharide binding to barley class II chitinase belonging to family GH19 (Hollis et al. 1997). The conformational changes induced by the oligosaccharide binding affect the environment surrounding tryptophan residues, thereby changing the tryptophan fluorescence intensity of the enzyme protein, which is obtained by excitation at 295 nm. By monitoring the fluorescence intensity, titration of an oligosaccharide solution can be performed to obtain a saturation curve for oligosaccharide binding. Binding data are usually analyzed by the Scatchard equation,

$$\Delta F = \Delta F_{\max} \cdot \frac{K_{\text{assoc}} \cdot [\text{S}]}{1 + K_{\text{assoc}} \cdot [\text{S}]}$$

or

$$\frac{\Delta F}{[\text{S}]} = -K_{\text{assoc}} \cdot (\Delta F - \Delta F_{\max})$$

where

ΔF and $\Delta F_{\max}$ represent the fluorescence change and its maximum, respectively
[S] is the free substrate concentration
K_{assoc} is the association constant

The dissociation constant K_D is equal to $1/K_{\text{assoc}}$. The fluorescence intensities were determined in the presence of different concentrations of the substrate, and the data were analyzed by least square curve fitting to obtain the values of $\Delta F_{\max}$ and K_{assoc} (K_D). The K_D values for $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$ toward barley class II chitinase were reported to be 43, 19, and 6 μM, respectively (Hollis et al. 1997). Thus, the longer the chain length of the ligand, the larger the affinity toward the enzyme. The K_{assoc} value can be converted to binding free energy change according to the equation,

$$\Delta G^{\circ} = -RT \cdot \ln(K_{\text{assoc}})$$

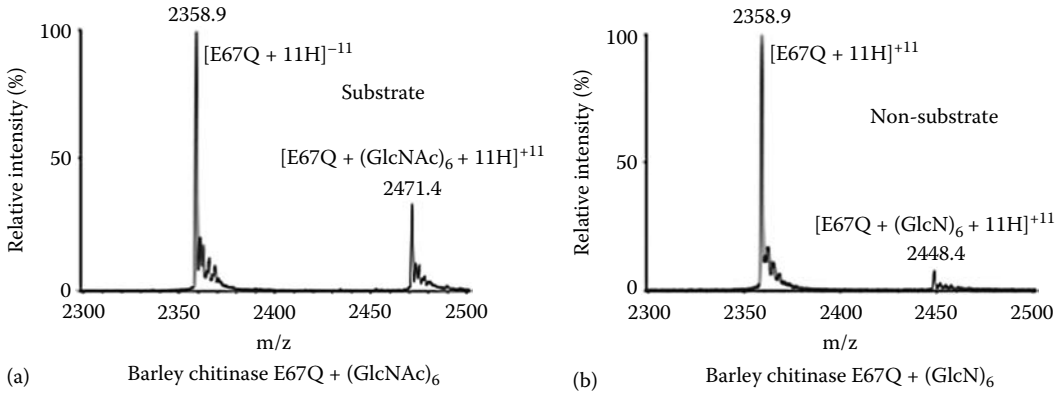

FIGURE 23.9 Mass spectra of E67Q barley chitinase and its complex. (a) Mass spectrum of E67Q barley chitinase (0.5 μM) and $(GlcNAc)_6$ (25 μM) in 10 mM ammonium acetate buffer pH 5.2, (b) Mass spectrum of E67Q barley chitinase (0.5 μM) and $(GlcN)_6$ (25 μM) in 10 mM ammonium acetate buffer pH 5.2.

where, ΔG^o, R, and T are the free energy change of binding, gas constant, and absolute temperature, respectively.

Recent developments in mass spectrometry have allowed us to determine oligosaccharide binding affinities to enzymes. Oligosaccharide binding experiments by mass spectrometry were successfully conducted using barley class II chitinase (Fukamizo et al. 2008). Electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) was used to detect the mass signal of the noncovalent complex of the chitinase and oligosaccharide. When the wild-type chitinase and $(GlcNAc)_6$ were used for the binding experiments, unfortunately, no meaningful MS spectrum was observed for the complex formation, probably due to the fast degradation of the substrate. So, the inactive mutant E67Q, in which the catalytic residue Glu67 has been mutated to Gln, was used for the binding experiments. A typical sprectrum is shown in Figure 23.9a, which clearly exhibits the free enzyme signal as well as the complexed enzyme signal. When complex formation was attempted using E67Q and the deacetylated derivative, $(GlcN)_6$, a small but significant signal derived from the complex was observed as shown in Figure 23.9b. Thus, the interaction of the binding site of the enzyme with $(GlcN)_6$ appears to be considerably weaker than that with $(GlcNAc)_6$, indicating importance of the *N*-acetyl groups in the enzyme–substrate interaction. Oligosaccharide titration was performed for K_D determination, while the enzyme concentration was kept constant. Figure 23.10 shows the Scatchard plot for $(GlcNAc)_3$ oligosaccharide binding to the E67Q mutant enzyme. From the slope of the lines, K_D was calculated to be 93 μM. For $(GlcNAc)_2$ and $(GlcNAc)_4$, the K_D values were 105 and 66 μM, respectively. The affinities obtained by ESI-TOF-MS were found to be lower than those obtained by fluorescence spectroscopy.

23.2.4 Estimation of the Subsite Affinities of Chitinases

Oligosaccharide molecules are able to bind to the enzyme-binding cleft in various productive binding modes, producing several oligosaccharides with lower polymerization degrees. This results in a complicated profile of the reaction time course. In addition, when a chitinase enzyme catalyzes a transglycosylation reaction, enzyme activity cannot simply be evaluated from the rate of substrate consumption or product formation (Fukamizo et al. 2001, Aguilera et al. 2003). In an investigation of the reaction of hen-egg white lysozyme toward oligosaccharide substrates, kinetic analysis of the time courses of substrate degradation and product formations were successfully performed using a kinetic model, in which the transglycosylation process was introduced in addition to the hydrolytic process and all possible binding modes were taken into consideration (Fukamizo et al. 1986a). This method has been applied to the chitinase-catalyzed hydrolysis (Honda and Fukamizo 1998,

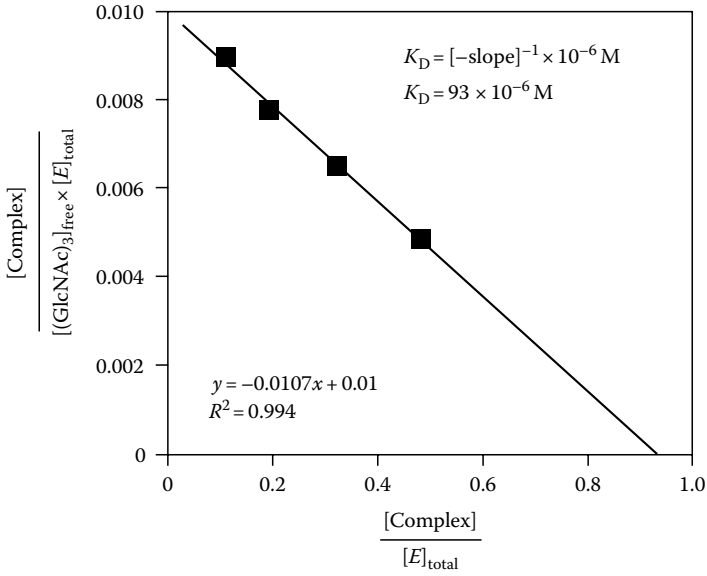

FIGURE 23.10 Scatchard plots for the binding of the $(GlcNAc)_3$ oligosaccharide to the E67Q mutant of barley chitinase. The data points were obtained from the signal intensities of the complex shown in the mass spectra. Conditions were the same as in Figure 23.9.

Fukamizo et al. 2001). The sugar residue affinities of individual subsites have been estimated by kinetic modeling of the reaction time course obtained by HPLC. A typical model scheme is shown in Figure 23.11. Based on the HPLC data of enzymatic hydrolysis of oligosaccharide substrates, the subsite structure can be estimated for individual enzymes. For example, the six subsites, −4, −3, −2, −1, +1, and +2, were assumed for rice class III chitinase (family GH-18, Figure 23.5), and −3, −2, −1, +1, +2, and +3 were assumed for rice class I chitinase (family GH-19, Figure 23.6). (The subsite nomenclature proposed by Davies et al. (1997) is employed in this chapter.) The binding constants for individual binding processes were calculated from the sum of the binding free energy changes (affinities) of individual subsites occupied with sugar residues. By assuming rapid attainment of equilibrium, the concentrations of the ES-complexes with all possible binding modes ($C_{n,i}$) were calculated based on the binding constants. The oligosaccharide substrate binds to the enzyme forming the $C_{n,i}$ complex, and the β-1,4-glycosidic linkage of the bound oligosaccharide is hydrolyzed with the rate constant k_{+1} to form the $B_{i,j}$ complex. The A_i complex is formed by releasing the hydrolytic fragment, and attacked by OH^- with the rate constant k_{+2} to complete hydrolysis. The A_i complex is also attacked by an oligosaccharide fragment with the rate constant k_{-1} to complete transglycosylation. For chitinases, which do not catalyze transglycosylation, the k_{-1} value was assumed to be zero. The processes represented by rate constants were modeled by simultaneous differential equations, which were numerically solved to obtain the theoretical time course of enzymatic hydrolysis (Fukamizo et al. 1986a; Honda and Fukamizo 1998). To estimate the individual subsite affinities, an optimization technique based on the modified Powell method (Kuhara et al. 1982) was employed using the cost function,

$$F = \Sigma\Sigma\left[(\text{GlcNAc})c_{n,i} - (\text{GlcNAc})e_{n,i}\right]^2 \tag{23.1}$$

where

e and *c* represent the experimental and calculated values, respectively
n is the size of the oligosaccharides
i the reaction time

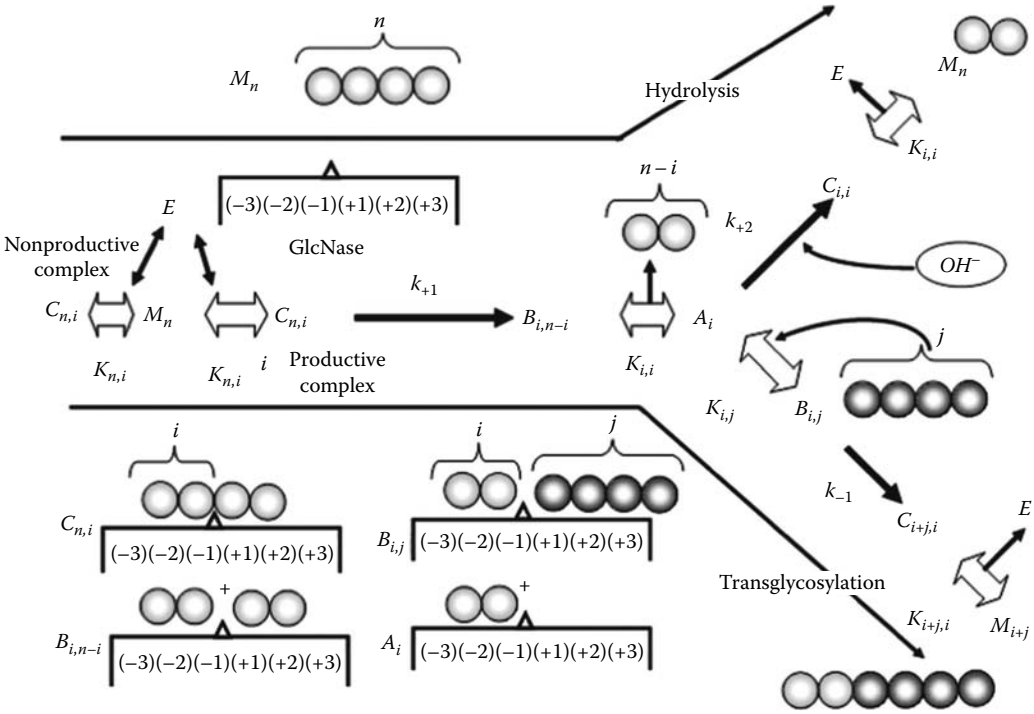

FIGURE 23.11 Reaction model scheme for chitinase-catalyzed hydrolysis and transglycosylation. E and M_n represent the enzyme and the substrate with a polymerization degree of n, respectively. Notations of individual complexed states, A_i, $B_{i,j}$, and $C_{n,i}$, are schematically described in the figure. The initial substrate binds to the enzyme with various binding modes to form $C_{n,i}$, which are then converted into $B_{i,n-i}$ through the cleavage of glycosidic linkage. The complex A_i is formed by releasing the oligosaccharide fragment bound to subsites with the positive numbers. The transglycosylation acceptor can bind again to subsites with the positive numbers, to form the complex $B_{i,j}$. Reaction parameters consist of three rate constants, k_{+1}, k_{-1}, and k_{+2}, and six binding free energy changes of individual subsites, (–3)~(+3). In the practical calculation, all possible binding modes are taken into consideration.

At first, the rate constants for glycosidic bond cleavage (k_{+1}) for oligosaccharides with various chain lengths were estimated from the maximal velocity data (V_{max}). Then the turnover numbers (k_{cat}) obtained were allocated to the k_{+1} values for individual oligosaccharide substrates, which were fixed in the modeling calculation. As reported by Masaki et al. (1981b), the values of k_{-1} and k_{+2} could not be determined independently, hence only the ratio k_{-1}/k_{+2} was determined based on the time-course data. A much higher value than that of k_{+1} is usually allocated to k_{+2}, and the k_{-1} value was optimized based on the time course. Using the constant values of k_{+1} and k_{+2}, the k_{-1} value (rate constant for transglycosylation) and the values of binding free energy changes of individual subsites (subsite affinities) were optimized based on the experimental time course. A typical example of the modeling calculation is shown in Figure 23.12. A class V chitinase (family GH-18) from cycad, *Cycas revoluta*, which catalyzes transglycosylation in addition to hydrolysis, was employed to hydrolyze the substrates $(GlcNAc)_4$ and $(GlcNAc)_6$. The profile of the experimental time courses was successfully reproduced by the modeling calculation as shown in the figure. The values of kinetic constants and the subsite affinities used for the modeling calculation are listed in Table 23.1. The transglycosylation reaction was found to take place as efficiently as hydrolysis in this enzymatic reaction. An identical set of subsite affinities of individual subsites could be used to reproduce both the time courses for $(GlcNAc)_4$ and $(GlcNAc)_6$ substrates, suggesting sufficient accuracy of the estimated values.

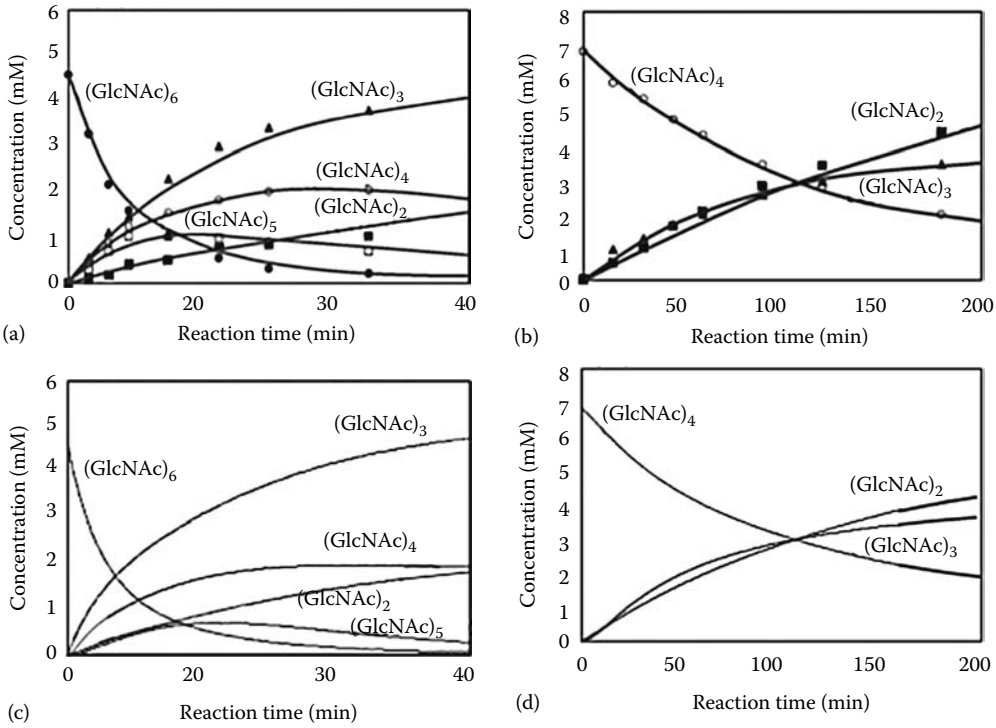

FIGURE 23.12 Theoretical calculation of the time courses of $(GlcNAc)_n$ ($n = 4$ and 6) hydrolysis and transglycosylation catalyzed by class V chitinase from cycad (*Cycas revoluta*). The reaction conditions were the same as in Figure 23.2. (a) Experimental time courses of the $(GlcNAc)_6$ degradation. (b) Experimental time courses of the $(GlcNAc)_4$ degradation. (c) Calculated time courses of the $(GlcNAc)_6$ degradation. (d) Calculated time courses of the $(GlcNAc)_4$ degradation. Based on these calculations, the affinities (binding free energy changes) of the individual subsites were estimated as listed in Table 23.1.

TABLE 23.1
The Values of Rate Constants and Binding Free Energy Changes for the Chitin Oligosaccharide Hydrolysis and Transglycosylation Catalyzed by Class V Chitinase from Cycad (*Cycas revolta*)

				Binding Free Energy Changes (kcal/mol)					
Substrate	k_{+1} (s^{-1})	k_{-1} (s^{-1})	k_{+2} (s^{-1})	**(−3)**	**(−2)**	**(−1)**	**(+1)**	**(+2)**	**(+3)**
$(GlcNac)_6$	120.0	220.0	200.0	−0.3	−4.5	+4.1	−0.3	−2.8	−3.2
$(GlcNac)_5$	120.0	220.0	200.0	−0.3	−4.5	+4.1	−0.3	−2.8	−3.2
$(GlcNac)_4$	220.0	220.0	200.0	−0.3	−4.5	+4.1	−0.3	−2.8	−3.2

23.3 CHITOSAN OLIGOSACCHARIDES

23.3.1 Preparation

The chitosan oligosaccharides [$(GlcN)_n$, $n = 1, 2, 3, \ldots$; Figure 23.13] can be prepared by acid hydrolysis of chitosan (Horowitz et al. 1957, Domard and Cartier, 1989), followed by separation using the Dowex 50w ion-exchanger (OH^- form), due to the cations (NH_3^+) attached to the C2 carbon of individual sugar residues. The chitosan hydrolysates were applied onto the Dowex column, and then

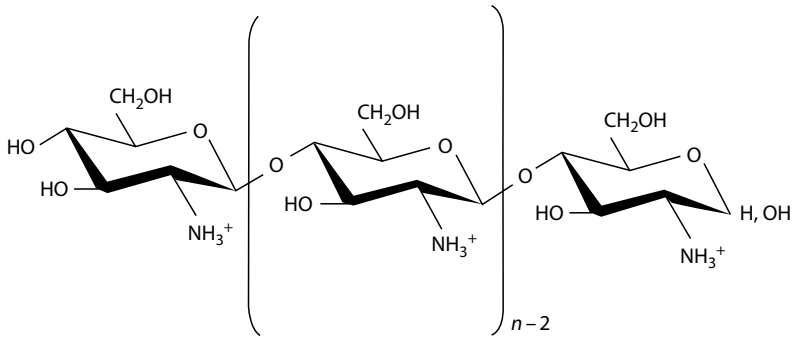

FIGURE 23.13 Chemical structure of chitosan, β-1,4-linked 2-amino-2-deoxy-D-glucopyranose polysaccharide.

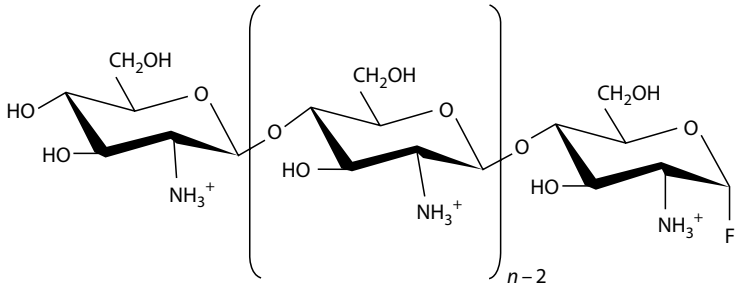

FIGURE 23.14 Chemical structure of β-1,4-linked 2-amino-2-deoxy-D-glucopyranose fluoride tetrasaccharide.

eluted with step-wise gradient of hydrochloric acid (0 → 2.5 M). A gel-filtration column (Cellulofine Gcl-25m) equilibrated with a buffer consisting of 0.25 M acetic acid: 0.05 M ammonium acetate (1:1, pH 4.0) was also used for the separation of chitosan oligosaccharides. The oligosaccharides in the effluents were determined by ninhydrin color detection at 570 nm. The reducing sugar method was also used for determination of individual oligosaccharides.

The fluorinated derivatives of the chitosan oligosaccharides were also obtained by fluorolysis of chitosan using hydrogen fluoride (Defaye et al. 1994). Gel-permeation chromatography of the fluorolysis products was performed using acetic acid–ammonium acetate (pH 4.0) as the eluent. These procedures yielded the α-glucopyranosyl fluorides of the chitosan oligosaccharides (Figure 23.14). The glucosyl fluorides were further hydrolyzed by aqueous perchloric acid, producing the chitosan oligosaccharides. These oligosaccharides were separated and purified by ion exchange chromatography or gel-filtration, as described above.

Nitrous acid treatment of chitosan also produced chitosan oligosaccharide derivatives, in which the reducing end residues had been converted into 2,5-anhydro-D-mannose (Tømmeraas et al. 2001). The molecular weight distribution was controlled by the molar ratio of nitrous acid to glucosamine residues (Allan and Peyron 1995). From the highly *N*-acetylated chitosan, treatment with an excess amount of nitrous acid produced fully *N*-acetylated chitin oligosaccharides in which the reducing end residue was 2,5-anhydro-D-mannose. Thus, the nitrous acid method is useful for obtaining oligosaccharides with a desired polymerization or *N*-acetylation degree. Enzymatic degradation of chitosan has also been conducted for obtaining chitosan oligosaccharides (Cheng and Li 2000, Choi et al. 2004).

23.3.2 Separation and Quantitative Determination

In analytical separation of chitosan oligosaccharides, the TLC method is most frequently used due to its highest feasibility. A solvent system consisting of *n*-propanol:28% ammonia:water (70:15:15)

is generally employed for the separation of the chitosan oligosaccharides using a silica gel plate (Silica gel 60, Merck). After the separation, oligosaccharides are visualized by spraying the plate with aniline–diphenylamine reagent (4 mL of aniline, 4 g of diphenylamine, 200 mL of acetone, and 30 mL of 85% phosphoric acid) and baking them at 180°C for 10 min. Visualization can be performed as well by spraying 0.1% ninhydrin in *n*-butanol-saturated water and by baking them at 110°C for 10 min. Chang and Hash (1979) tried to use a conventional amino acid analyzer for facilitating the determination of chitosan oligosaccharides. After the lag of 16 years, an efficient HPLC system for the separation of chitosan oligosaccharides was first reported by Fukamizo et al. (1995), who used a gel-filtration column of TSK-GEL G2000PW (Tosoh) and revealed its effectiveness for quantitative determination of chitosan oligosaccharides. In the gel-filtration HPLC, an ionic strength of the elution solvent should be increased to suppress the electrostatic interaction of chitosan oligosaccharides. A partition HPLC column (TSK-GEL NH_2-60, Tosoh) has also been used for the separation (Lee et al. 2006). Elution was conducted with 60% acetonitrile at a flow rate of 0.8 mL/min. In all HPLC systems, a refractive index detector was used to detect the oligosaccharides. Separation profiles for TLC and HPLC appear in Section 23.3.3.

Recent progress in mass spectrometric techniques has enabled more effective determination of chitosan oligosaccharides. Recently, Trombotto et al. (2008) reported the MALDI-TOF mass spectra of chitosan oligosaccharides obtained by acid hydrolysis of polysaccharide chitosan. Oligomers with different polymerization degrees were clearly resolved, and the peak-to-peak mass difference was 161 mass units, which matches the GlcN repeating unit ($C_6H_{11}O_4N$). Thus, the distribution of polymerization degree of the acid hydrolysate was directly evaluated from the spectra. The method is now regarded as a powerful technique for determination of chitosan oligosaccharides.

23.3.3 Action of Endo-Splitting Chitosanase toward Chitosan Oligosaccharides

Among the endo-splitting chitosanases, family GH46 enzyme from *Streptomyces* sp. N174 was most intensively studied for its structure and function (Fukamizo and Brzezinski 1997). As in the case of chitinases, monitoring the enzymatic hydrolysis of oligosaccharide substrate affords useful information on the mode of action of the chitosanase. TLC separation of the products from the chitosanase hydrolysis of chitosan oligosaccharides gave a characteristic profile showing a major product $(GlcNAc)_3$ and minor products $(GlcNAc)_2$ and $(GlcNAc)_4$ (data not shown). When the fluorinated derivatives of the chitosan oligosaccharides [$(GlcN)_n$-F, n = 1–6], in which the reducing end residues had been converted into glycosyl fluoride, were used as the chitosanase substrates, the mode of enzyme action was deduced from the TLC profiles shown in Figures 23.15 and 23.16. The enzyme hydrolyzed $(GlcN)_5$-F, producing $(GlcN)_2$ + $(GlcN)_3$-F and $(GlcN)_3$ + $(GlcN)_2$-F. The reaction producing $(GlcN)_2$ + $(GlcN)_3$-F appears to be more intensive than producing $(GlcN)_3$ + $(GlcN)_2$-F (Figure 23.15). From the $(GlcN)_6$-F substrate, the enzyme produced $(GlcN)_3$ + $(GlcN)_3$-F predominantly, and the amount of $(GlcN)_2$ produced was larger than that of $(GlcN)_4$ produced, indicating that the production of $(GlcN)_2$ + $(GlcN)_4$-F is more intensive than that of $(GlcN)_4$ + $(GlcN)_2$-F (Figure 23.16). From these results, mode of hydrolysis of $(GlcN)_n$-F could be deduced as indicated in the individual figures. More quantitative data were obtained by gel-filtration HPLC. The time-dependent HPLC profiles of $(GlcN)_n$ hydrolysis by the chitosanase are shown in Figure 23.17a. The peak areas of the individual oligosaccharides were converted into molar concentrations, which were plotted against the reaction time to obtain the reaction time course shown in Figure 23.17b. The result was consistent with the data obtained by TLC (Figure 23.16), which showed the hydrolysis of $(GlcN)_6$-F predominantly into $(GlcN)_3$ + $(GlcN)_3$-F. These results suggest that this chitosanase is an endo-splitting enzyme possessing a long-extended substrate-binding cleft. Since the $(GlcN)_6$ substrate is symmetrically hydrolyzed, the catalytic residues are likely located in the central portion of the binding cleft. In fact, the x-ray crystal structure of the enzyme revealed that the enzyme has an opened binding cleft that can accommodate several sugar residues (Marcotte et al. 1996).

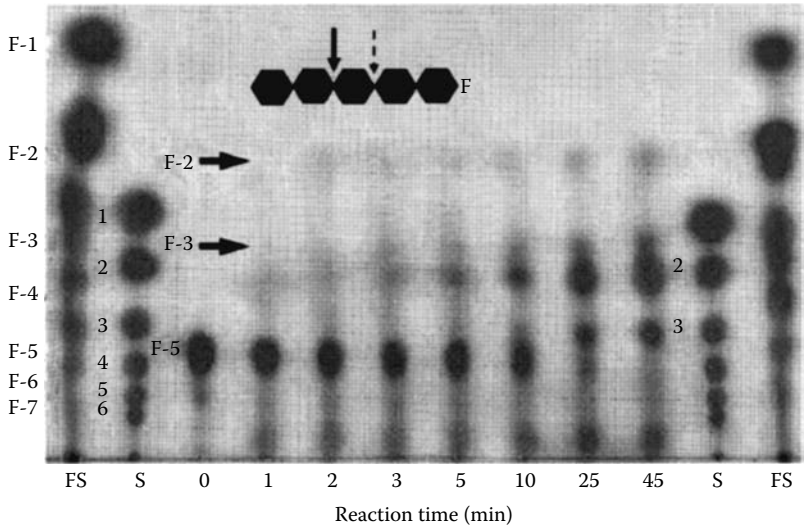

FIGURE 23.15 TLC profile showing $(GlcN)_5$-F hydrolysis catalyzed by *Streptomyces* sp. N174 chitosanase. The enzymatic reaction was performed in 50 mM sodium acetate buffer pH 5.0. Concentrations of the enzyme and the substrate were 1 μM and 2 mM, respectively. 1–6, GlcN–$(GlcN)_6$; F-1–F-7, GlcN-F–$(GlcN)_7$-F; FS, oligosaccharide mixture of GlcN-F–$(GlcN)_7$-F; S, oligosaccharide mixture of GlcN–$(GlcN)_6$.

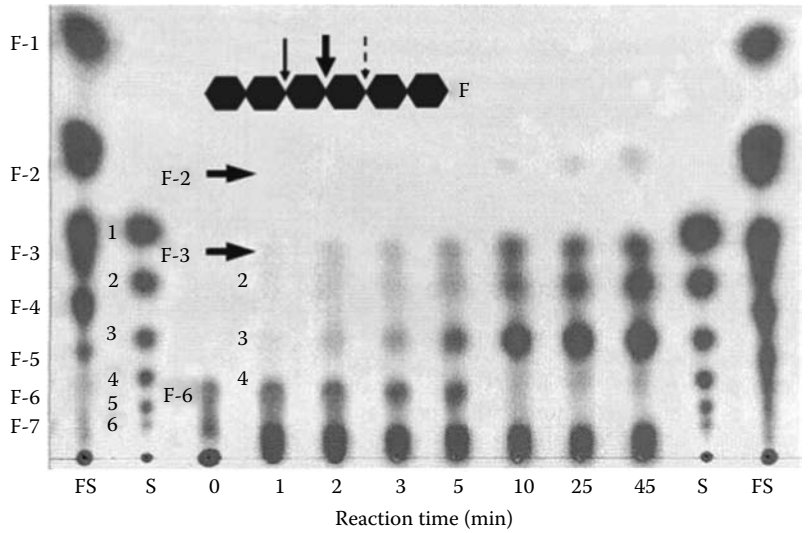

FIGURE 23.16 TLC profile showing $(GlcN)_6$-F hydrolysis catalyzed by *Streptomyces* sp. N174 chitosanase. Reaction conditions were the same as in Figure 23.15. Abbreviations used are also the same as in Figure 23.15.

From a site-directed mutagenesis study, the catalytic residues were found to be Glu22 and Asp40 (Boucher et al. 1995), which are separately located in the midst of the binding cleft.

The enzymatic hydrolysis of $(GlcN)_6$ was also monitored by ^{1}H NMR spectroscopy. The anomeric proton signals of the reducing end of the substrate were gradually enhanced due to the hydrolysis of glycosidic linkages, as shown in Figure 23.18. The increase in the α-anomer signal was more intense than that in the β-anomer signal. The *Streptomyces* chitosanase belonging to family GH46 hydrolyzes β-1,4-glycosidic linkage, producing the α-anomer; i.e., it is an inverting enzyme

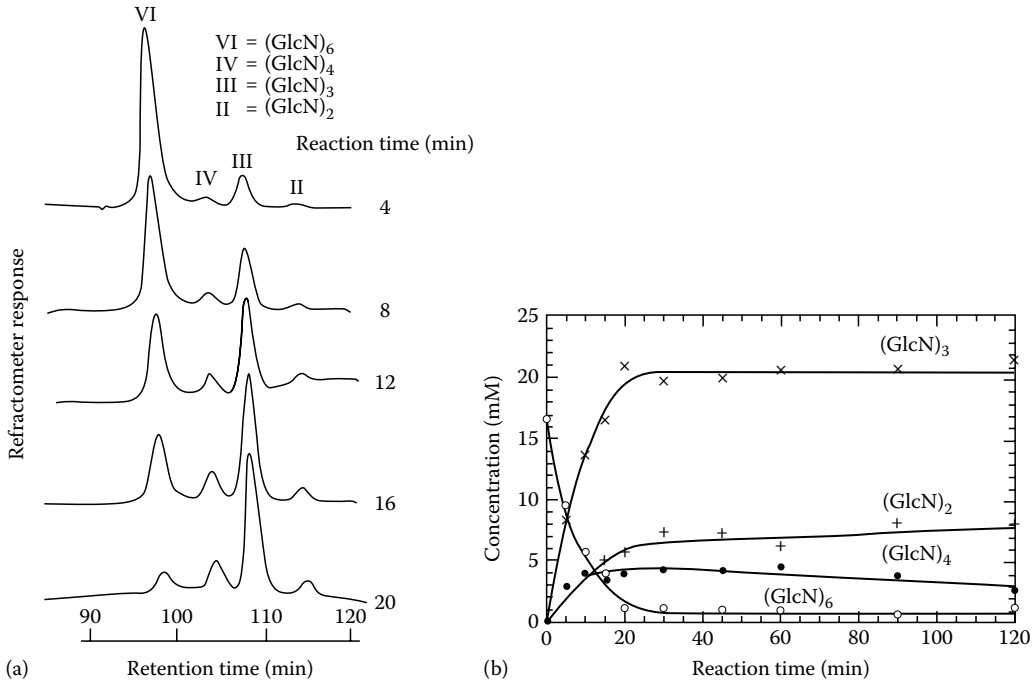

FIGURE 23.17 HPLC profile showing the $(GlcN)_6$ hydrolysis catalyzed by *Streptomyces* sp. N174 chitosanase (a), and the time course of the reaction (b). A gel-filtration column (7.5 mm × 600 mm) of TSK-GEL G2000PW was used for the separation, and elution was performed with 0.5 M NaCl at room temperature at a flow rate of 0.3 mL/min. $(GlcN)_n$ were detected with a differential refractometer. The enzyme (0.19 nmol) was added to 0.5 mL of 16.6 mM $(GlcN)_6$ solution dissolved in 50 mM sodium acetate buffer, pH 5.5, and the reaction was carried at 40°C. The individual oligosaccharide concentrations calculated based on the HPLC profiles were plotted against the reaction time to obtain the reaction time course.

(Fukamizo et al. 1995). All of these results support the catalytic mechanism shown in Figure 23.19. Glu22 donates a proton to the glycosyl oxygen atom to split the linkage, and then the water molecule activated by Asp40 attacks the C1 carbon of the −1 sugar residue. A similar catalytic reaction takes place during the chitin oligosaccharide hydrolysis catalyzed by family GH-19 chitinases, such as rice class I chitinase (Figure 23.6). A chitosanase belonging to family GH5 was isolated from *Streptomyces griseus* HUT 6037, which found to produce β-anomer by ^{1}H NMR spectroscopy (Tanabe et al. 2003). This was the first discovery of a retaining endochitosanase.

The time course of chitosanase hydrolysis was also determined by real-time ESI-MS (Dennhart et al. 2008), as shown in Figure 23.20. The result is consistent with that obtained by HPLC (Figure 23.17). In the real-time ESI-MS for chitosanase hydrolysis, similar advantages were found as in the case of the chitinase-catalyzed hydrolysis described in Section 23.2.2, i.e., the amounts of enzyme and substrate used were much lower than those used for HPLC determination, and the real-time ESI-MS method was much more faster.

23.3.4 Oligosaccharide Binding to Chitosanases

The chitosan oligosaccharide binding to chitosanase was first reported by Honda et al. (1997). They indirectly evaluated the binding ability of *Streptomyces* sp. N174 chitosanase from the elevation of its unfolding transition temperature (ΔT_m). The unfolding transition curves were determined by CD or fluorescence spectroscopy. Figure 23.21 shows the unfolding curves obtained by monitoring tryptophan fluorescence intensity. For facilitating the comparison, the fraction of unfolded protein

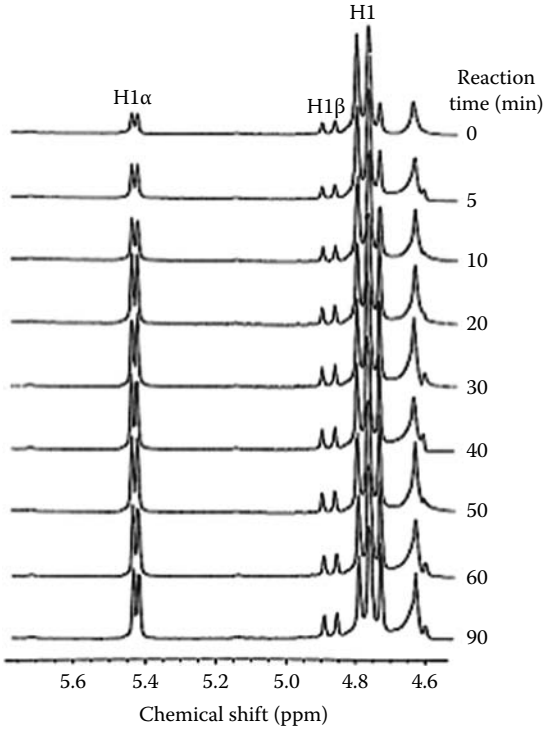

FIGURE 23.18 ^{1}H-NMR spectra showing the $(GlcN)_6$ hydrolysis catalyzed by *Streptomyces* sp. N174 chitosanase. Hlα, Hlβ and H1 indicate the signals derived from the anomeric protons of the reducing-end residue and those of the other GlcN residues respectively. The enzyme (0.36 nmol) was mixed with 0.5 mL of 8.3 mM $(GlcN)_6$ solution dissolved in 10 mM deuterated sodium acetate buffer.

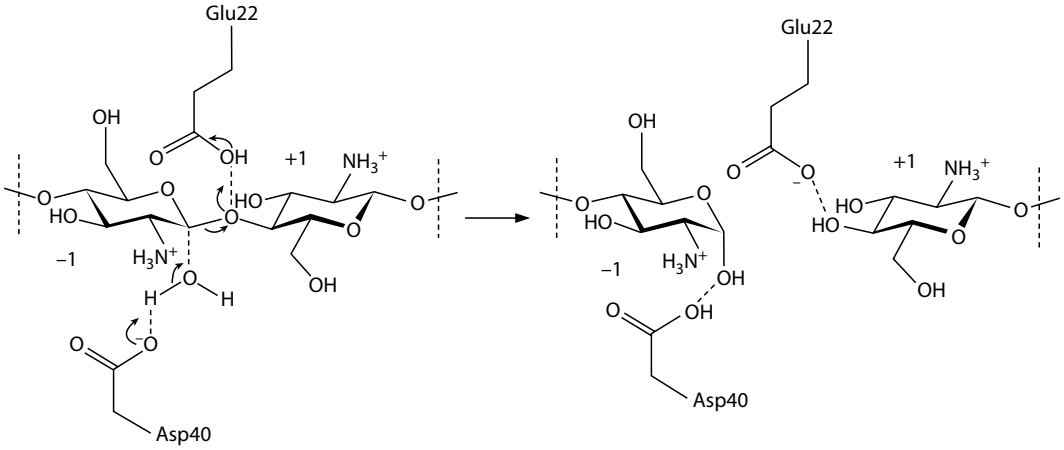

FIGURE 23.19 Catalytic mechanism of chitosan oligosaccharide hydrolysis by *Streptomyces* sp. N174 chitosanase.

was calculated for individual temperatures based on the changes in fluorescence intensity. In the presence of the chitosan oligosaccharide, the ligand binding stabilizes the enzyme protein structure resulting in T_m elevation. Interestingly, the higher the polymerization degree of the oligosaccharide added, the larger the T_m elevation, as shown in the figure. This indicates that the binding data obtained by this method are semiquantitative. When the Asp57-mutated chitosanase (D57A) was

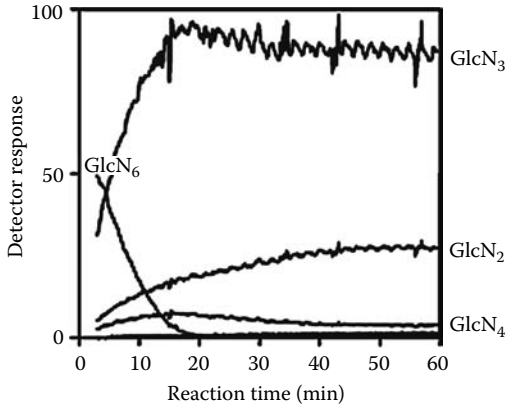

FIGURE 23.20 Real-time ESI-MS monitoring of $(GlcN)_6$ hydrolysis catalyzed by *Streptomyces* sp. N174 chitosanase. The enzymatic reactions were carried out in 10 mM ammonium acetate-containing aqueous solutions pH 5.2 at 20°C. Concentrations of the enzyme and the substrate were 0.50 and 25.0 μM, respectively. Time-course data were corrected with ionization factors.

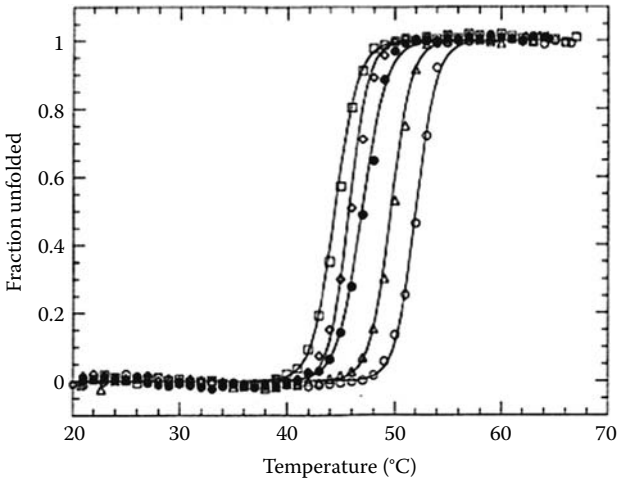

FIGURE 23.21 Thermal unfolding curves of the E22Q mutant of *Streptomyces* sp. N174 chitosanase in the presence of $(GlcN)_n$. Each enzyme solution was dissolved in 50 mM sodium phosphate buffer pH 7.0, and the tryptophan fluorescence intensity was monitored at 330 nm with excitation wavelength at 295 nm. The protein concentration was 3.2 μM and the concentration of each oligosaccharide added was 0.36 mM. The symbols represent □, E22Q; ◇, E22Q + $(GlcN)_3$; ●, E22Q + $(GlcN)_4$; △, E22Q + $(GlcN)_5$; and ○, E22Q + $(GlcN)_6$. The solid lines indicate the theoretical unfolding curves obtained with assuming the two-state transition mechanism.

used for binding experiments, no T_m elevation was observed, indicating that Asp57 is important for oligosaccharide binding (Tremblay et al. 2001).

The fluorescence spectrum of chitosanase was also used for determining the oligosaccharide binding to the enzyme (Fukamizo et al. 2005, Katsumi et al. 2005). Trp fluorescence intensity obtained by the excitation at 295 nm UV light was quenched by the addition of chitosan oligosaccharides, and the quenching effect was clearly dependent upon the amount of oligosaccharide added. Scatchard plots of the changes in fluorescence intensity shown in Figure 23.22 exhibited biphasic profiles for $(GlcN)_2$ and $(GlcN)_3$ binding to the chitosanase. The biphasic profiles indicate that the

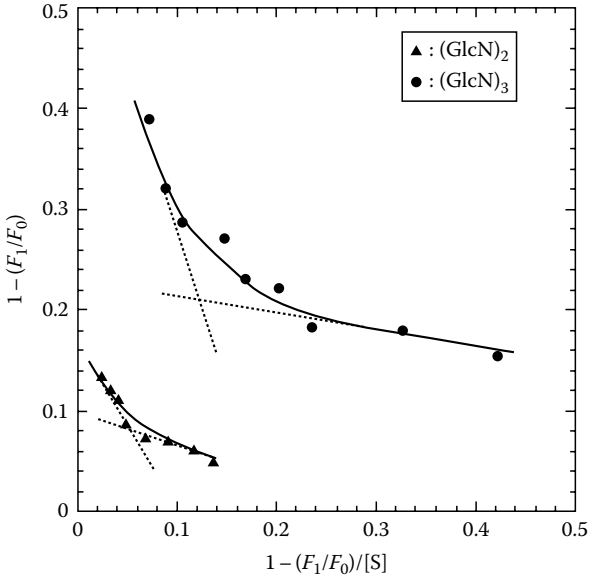

FIGURE 23.22 Scatchard plots of the fluorescence quenching induced by oligosaccharide binding to *Streptomyces* sp. N174 chitosanase. The chitosanase was dialyzed against 0.06 M Tris–HCl buffer, pH 7.0, containing 0.1 M NaCl, and then the spectra were recorded with a Hitachi F-3010 spectrofluorometer. The fluorescence intensity at 330 nm obtained by excitation at 295 nm was used for analyzing the oligosaccharide binding. Enzyme concentration was 2.5 μM. Symbols are ●, $(GlcN)_3$; and ▲, $(GlcN)_2$. The association constants were obtained from the slopes of the lines, and are listed in Table 23.2.

oligosaccharides bind to the enzyme with two different binding modes (a high affinity-binding mode and a low affinity-binding mode). Fluorescence analysis was employed to evaluate the mutation effect of E197A and D201A on the oligosaccharide binding ability of the chitosanase. When the mutant E197A was used for the binding experiments, the Scatchard plot was monophasic, and the slope of the line corresponded to that of the low-affinity mode of the wild-type chitosanase. The Scatchard plot for D201A was also monophasic, but the slope corresponded to that of the high-affinity mode of the wild-type. Thus, the acidic amino acids Glu197 and Asp201 appear to be responsible for oligosaccharide binding to the high-affinity site and the low-affinity site, respectively (Katsumi et al. 2005). The association constants and binding free energy changes obtained by these analyses are listed in Table 23.2.

TABLE 23.2
Association Constants and Binding Free Energy Changes for $(GlcN)_2$ or $(GlcN)_3$ Binding to *Streptomyces* sp. N174 Chitosanase

		High Affinity Site		Low Affinity Site	
Chitosanase	**Saccharide**	K_{assoc} (M^{-1})	ΔG_u (kcal/mol)	K_{assoc} (M^{-1})	ΔG_u (kcal/mol)
Wild type	$(GlcN)_2$	2941	–6.9	605	–6.0
	$(GlcN)_3$	6410	–7.4	328	–5.7
E197A	$(GlcN)_3$	n.d.	n.d.	318	–5.6
D201A	$(GlcN)_3$	4710	–7.2	n.d.	n.d.

Note: n.d., not determined.

Surface plasmon resonance (SPR) measurements were also performed to evaluate binding ability. A BIAcore 2000 system equipped with a sensorchip (CM5), to which the chitosanase protein was immobilized by the amine coupling method, was used for the SPR measurements. After stabilizing the detector response for 2 min, chitosan oligosaccharide solution was injected over the sensorchip at a flow rate of 10 μL/min. The injection was continued for 7 min and then the buffer solution was injected for dissociation from the sensorchip. The results are shown in Figure 23.23. When the GlcN monomer was injected over the sensorchip, the detector did not respond at all. When $(GlcN)_2$ was used instead of GlcN, significant responses were obtained, and this clearly dependent upon the saccharide concentration. Similarly, the injection of $(GlcN)_3$ resulted in a concentration-dependent profile. However, the responses were considerably enhanced compared with those obtained for $(GlcN)_2$. Further enhancement was observed in the response to $(GlcN)_4$. For $(GlcN)_5$ and $(GlcN)_6$, however, the profiles were similar to that of $(GlcN)_4$. These oligosaccharides with longer chain lengths might be hydrolyzed at least partly by the immobilized chitosanase. From GlcN to $(GlcN)_4$, the higher the polymerization degree of the chitosan oligosaccharide, the stronger its binding interaction with chitosanase. SPR is also a powerful tool for determining the oligosaccharide–chitosanase interaction.

23.4 PARTIALLY *N*-ACETYLATED CHITOSAN OLIGOSACCHARIDES

23.4.1 Preparation and Structural Identification

Mono-*N*-acetylated chitobiose, in which the reducing end residue is *N*-acetylated, was first isolated from the chitinase digestion products of insect cuticle chitin, and identified by Fukamizo et al. (1986b). The structural identification was performed by ^{1}H- and ^{13}C-NMR spectroscopy. Mitsutomi and coworkers isolated several types of partially *N*-acetylated chitosan oligosaccharides from chitinase/chitosanase hydrolysates of partially *N*-acetylated chitosan (Mitsutomi et al. 1990, Ohtakara et al. 1990, Izume et al. 1992). In a similar manner, Fukamizo et al. (1995) produced the partially *N*-acetylated chitosan oligosaccharides, which were successfully purified by cation exchange column chromatography using CM-Sephadex C-25. A typical profile of the chromatographic separation is shown in Figure 23.24. From S1 to S3, individual fractions were identified to be mono-*N*-deacetylated chitin oligosaccharides with polymerization degree of 4, 3, and 2, respectively. From S6 to S8, individual fractions were identified to be di-*N*-deacetylated chitin oligosaccharides with polymerization degree of 5, 4, and 3, respectively. Obviously, the elution positions of the individual oligosaccharides are clearly dependent upon the number of deacetylated residues as well as the polymerization degree. This allows the efficient separation of hetero-oligosaccharides consisting of GlcNAc and GlcN. Sequencing of partially *N*-acetylated chitosan oligosaccharides was conducted by an enzymatic digestion technique, using *exo*-β-*N*-acetylglucosaminidase and *exo*-β-glucosaminidase, which split off the nonreducing end *N*-acetylglucosamine and glucosamine residues, respectively. Successive use of these enzymes allows the determination of an oligosaccharide sequence (Mitsutomi et al. 1990, 1995). However, NMR spectroscopy and mass spectrometry have been also used for sequence determination (Fukamizo et al. 1991). Since information obtained by NMR spectroscopy is restricted to the sugar residues of both terminal ends and their nearest neighbors, there is some limitation in chain length of the oligosaccharides that can undergo sequence determination by NMR spectroscopy. On the other hand, sequence determination for longer chain-length oligosaccharides has now become possible using mass spectrometry. Especially, matrix-assisted laser desorption ionization postsource decay mass spectrometry (MALDI-PSD MS) was successfully applied to the sequence determination (Bahrke et al. 2002).

23.4.2 Mode of Action of Enzymes toward Partially *N*-Acetylated Chitosan

The enzyme digestion of partially *N*-acetylated chitosan described in Section 23.4.1. provided information on the mode of action of chitinases/chitosanases. The sugar sequences at the reducing and

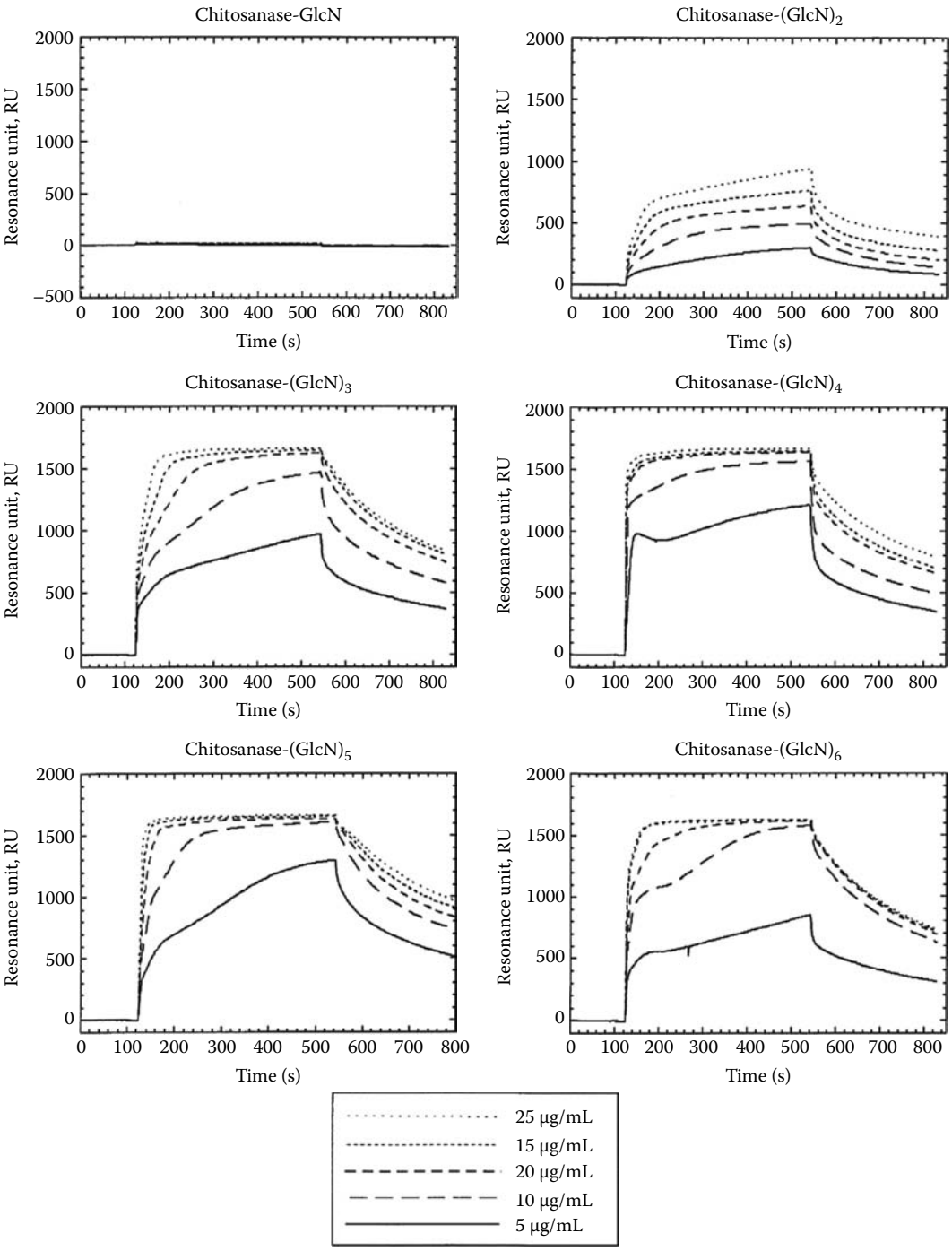

FIGURE 23.23 SPR sensorgrams showing interactions between *Streptomyces* sp. N174 chitosanase and $(GlcN)_n$. A BIAcore 2000 (BIAcore AB, Uppsala, Sweden) system was used to determine the interaction. The chitosanase protein was immobilized onto a CM5 sensorchip by the amine coupling method. The individual oligosaccharides were dissolved in 10 mM sodium acetate buffer pH 5.0, and the solutions were used as analytes.

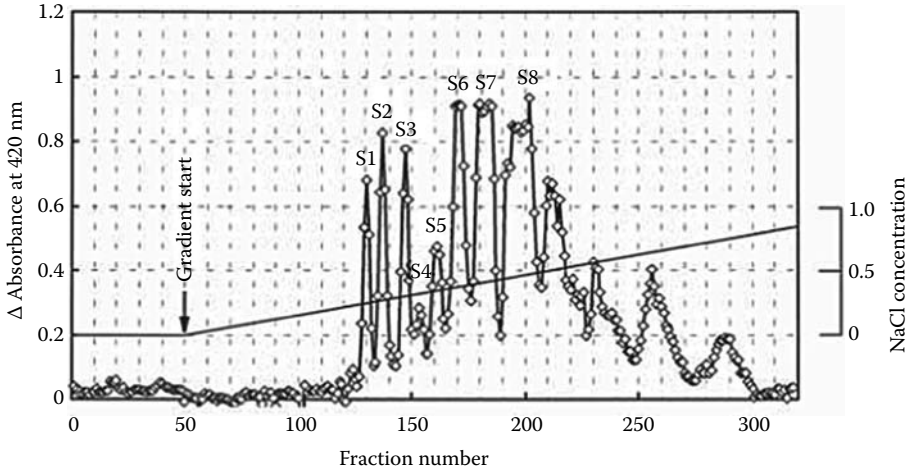

FIGURE 23.24 CM-Sephadex C-25 column (2.5 × 45 cm) chromatography of hetero-oligosaccharide products from the incubation of partially *N*-acetylated chitosan with *Streptomyces* sp. N174 chitosanase. The chitosanase hydrolysate was fractionated on a charcoal column, and the adsorbed fraction was applied to this column. Elution was performed with 10 mM sodium acetate buffer, pH 5.0. Linear gradient elution with NaCl was started at fraction number 50. The individual peak fractions were successfully identified by ^{1}H- and ^{13}C-NMR spectroscopy as follows: S1, GlcN–GlcNAc–GlcNAc–GlcNAc; S2, GlcN–GlcNAc–GlcNAc; S3, GlcN–GlcNAc; S6, GlcN–GlcN–GlcNAc–GlcNAc–GlcNAc; S7, GlcN–GlcN–GlcNAc–GlcNAc; S8, GlcN–GlcN–GlcNAc.

nonreducing ends of the oligosaccharide products were determined by ^{1}H and ^{13}C NMR spectroscopy. Based on these sequences, the specificity of the individual subsites (−2, −1, +1, and +2) toward the GlcNAc or GlcN residue has been deduced for bacterial and plant chitinases (Sorbotten et al. 2005, Sasaki et al. 2006, Heggset et al. 2009). Figure 23.25 shows the anomeric proton region of the ^{1}H NMR spectra of the oligosaccharide products obtained from rice chitinase digestion of partially *N*-acetylated chitosan. When the rice class III chitinase belonging to family GH18 was used for chitosan digestion, the α-anomer signal of GlcNAc (5.19 ppm) was clearly observed, whereas the GlcN signal was not detected at all. This indicates that the reducing end residues of oligosaccharide products are exclusively GlcNAc. Information on the nearest neighbors of the reducing end residues was found in the reducing end β-anomer signal at 4.68–4.76 ppm, which was separated into two doublet signals according to the neighboring residue; i.e., the reducing end β-signal of –GlcN–GlcNAc was observed at 4.75 ppm, while the signal of –GlcNAc–GlcNAc was seen at 4.70 ppm. The ratio between the two signal intensities was similar to the ratio between the GlcNAc- and GlcN-units (F_A) in the substrate used (Figure 25a and b). This is expected when the enzyme is absolutely specific to the GlcNAc units at subsite −1 but not specific to subsite −2. When the rice class I chitinase belonging to family GH19 was used instead of the class III chitinase, similarly, the newly produced reducing end was exclusively GlcNAc (Figure 23.25c, 5.19 ppm). As in the case of the class III enzyme, the class I enzyme preferentially binds to the GlcNAc units at subsite −1. However, the doublet signal of β-anomer of the reducing end GlcNAc was found only at 4.70 ppm, and was not split in any of the chitosan substrates with 0.32 and 0.64 of F_A (fraction of acetylated units) (Figure 23.25c and d). These results indicate that the nearest neighbor to the reducing end GlcNAc was exclusively GlcNAc. Thus, the class I enzyme is highly specific to GlcNAc at both subsites −1 and −2.

Information on the nonreducing end residues of the enzymatic products was obtained from the C5 and C3 regions of the ^{13}C NMR spectrum (Figure 23.26). The C5 signals of GlcNAc and GlcN units at nonreducing ends were detected at 78.4 and 79.0 ppm, respectively (Vårum et al. 1996). When the chitosan with F_A = 0.64 was digested by the rice class III chitinase, the signal intensities of both GlcNAc and GlcN increased with progress of the enzymatic reaction (Figure 23.26a).

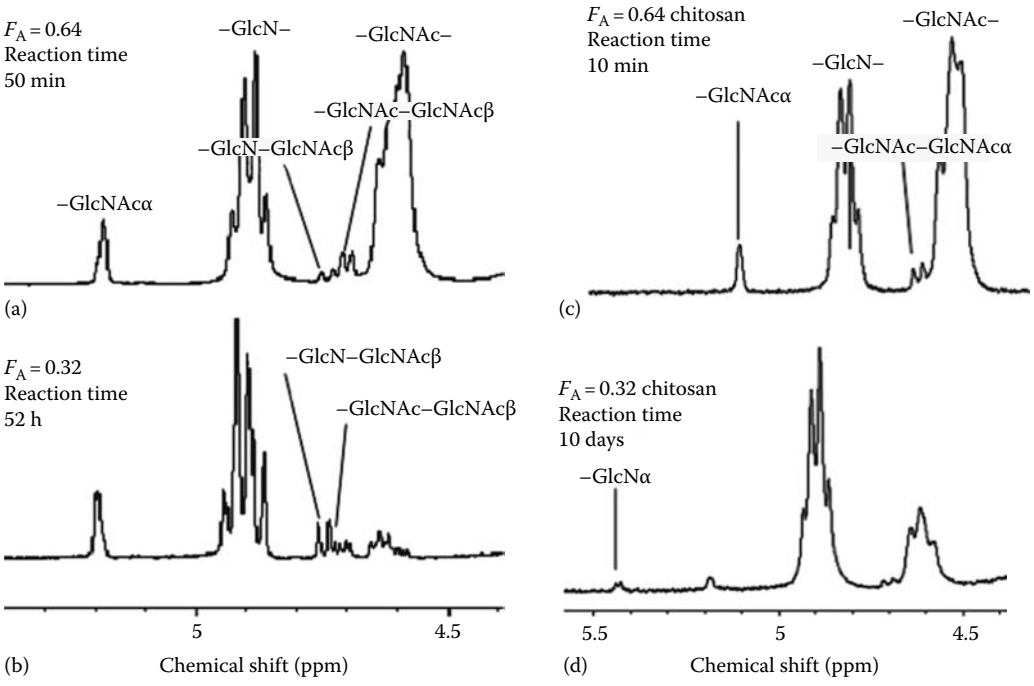

FIGURE 23.25 Anomeric proton region of ^{1}H-NMR spectra of rice chitinase products from chitosans with F_A (fraction of acetylated units)= 0.64 and F_A = 0.32. The chitosan with F_A = 0.64 (8.85 mg/mL) was hydrolyzed by 2 μM rice class III chitinase for 20 min (a), and the chitosan with F_A = 0.32 (12.5 mg/mL) was hydrolyzed by the enzyme (2 μM) for 52 h (b) at 40°C. The chitosan with F_A = 0.64 (8.85 mg/mL) was hydrolyzed by 0.002 μM rice class I chitinase for 5 min (c), and the chitosan with F_A = 0.32 (12.5 mg/mL) was hydrolyzed by the same enzyme (0.2 μM) for 10 days at 40°C (d).

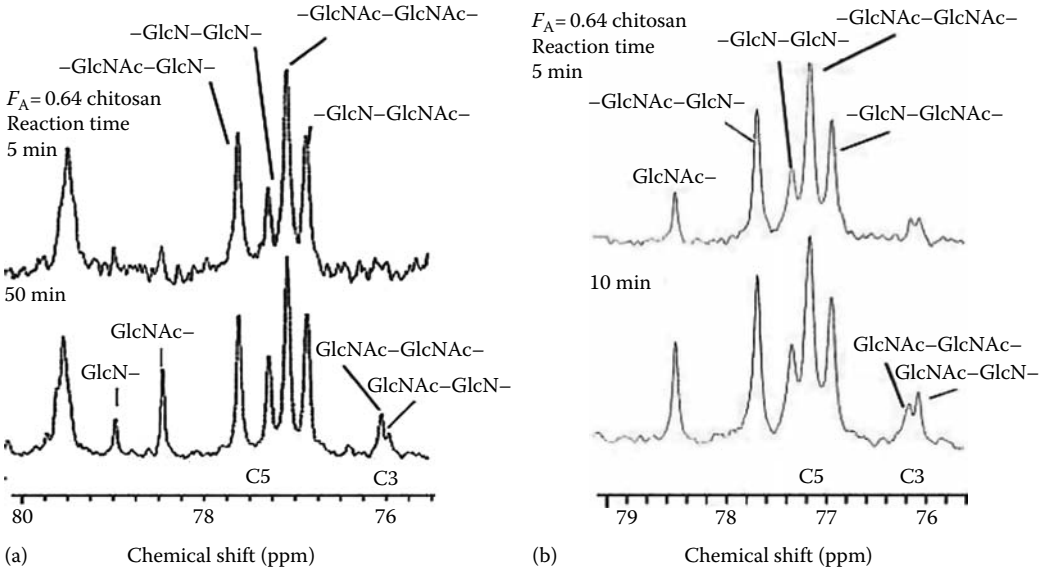

FIGURE 23.26 C5 and C3 regions of ^{13}C-NMR spectra of rice chitinase products from chitosan with F_A = 0.64. (a) The chitosan with F_A = 0.64 (8.85 mg/mL) was hydrolyzed by 2 μM rice class III chitinase for 5 min and 50 min at 40°C. (b) The chitosan with F_A = 0.64 (8.85 mg/mL) was hydrolyzed by 0.002 μM rice class I chitinase for 5 min and by the same enzyme (0.02 μM) for 10 min at 40°C.

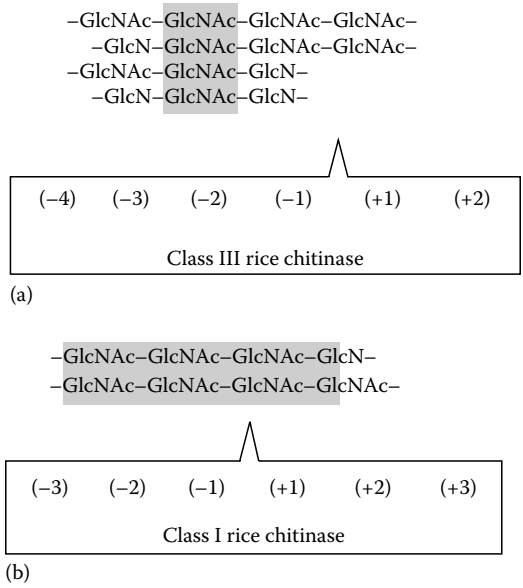

FIGURE 23.27 Sugar recognition specificity of class I and III chitinases from rice, *Oryza sativa* L. (a) class III and (b) class I.

The ratio between the signal intensities of GlcN- and GlcNAc-units was approximately 2:3 (0.67), which is similar to the F_A value of the chitosan substrate (0.64). The nonreducing end C3 signal of the GlcNAc unit was found to split into two signals (76.0 and 76.1 ppm) depending upon the neighboring residue (GlcNAc–GlcNAc– or GlcNAc–GlcN–), indicating that both GlcNAc and GlcN units were found as the nearest neighbor to the nonreducing end GlcNAc-unit (Figure 23.26a). Therefore, subsites +1 and +2 are not specific to the GlcNAc- or GlcN-unit. When the rice class I chitinase was used instead of the class III enzyme, the C5 signal of the nonreducing end GlcNAc was found at 78.5 ppm, whereas the signal of the nonreducing end GlcN was not detected in the corresponding position (79.0 ppm), as shown in Figure 23.26b. Thus, the nonreducing ends of the products were exclusively GlcNAc. In the nonreducing end C3 region (76.0 and 76.1 ppm), the intensity of GlcNAc–GlcN– was higher than that of GlcNAc–GlcNAc– (Figure 23.26b). Since the fraction of acetylated units was higher than that of deacetylated units in the substrate chitosan, the class I enzyme has a low but significant preference toward the GlcN units at subsite +2. Finally, the class I enzyme specifically binds to GlcNAc units at subsite +1 but has a low but significant preference for GlcN units at subsite +2.

All of these data are summarized in Figure 23.27. Among the contiguous subsites from –2 to +2 of the class III enzyme, only subsite –1 was found to be specific to a GlcNAc residue, while the three contiguous subsites –2, –1, and +1 of the class I enzyme are specific to three consecutive GlcNAc residues of the substrate.

23.4.3 Enzymatic Hydrolysis of Partially *N*-Acetylated Chitosan Oligosaccharides

Brzezinski and his coworkers isolated *exo*-β-glucosaminidase/*exo*-chitosanse from the culture supernatant of *Amycolatopsis orientalis* (Côté et al. 2006). They also cloned and sequenced the enzyme gene and produced a recombinant enzyme protein. The mode of action of the enzyme was investigated using mono-*N*-acetylated chitosan tetrasaccharides (Fukamizo et al. 2006). The enzymatic reaction was monitored by a gel-filtration HPLC using the detection of UV absorption at 215 nm originating from the *N*-acetyl groups of the substrate and its products. Time-dependent

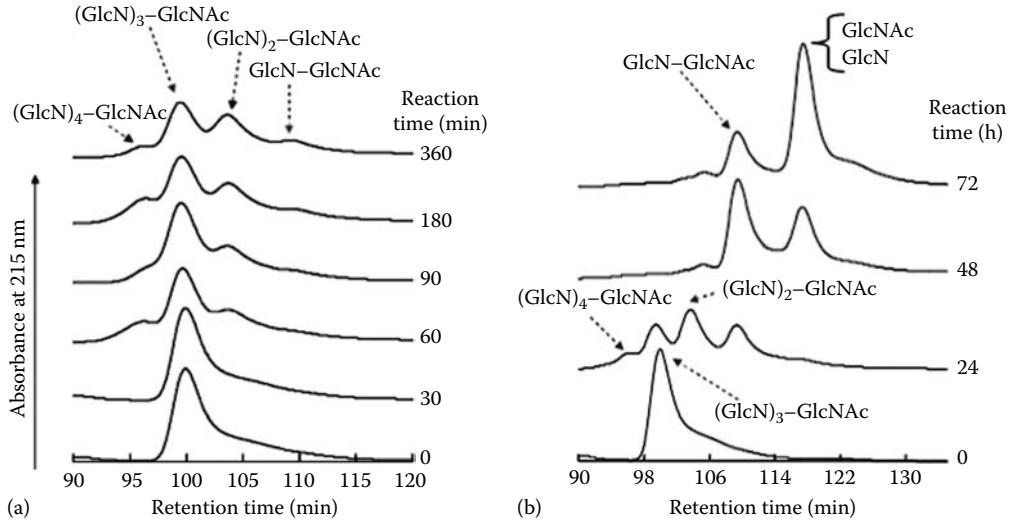

FIGURE 23.28 HPLC profiles of the products obtained from *exo*-β-glucosaminidase hydrolysis of $(GlcN)_3$–GlcNAc. The enzymatic reaction was performed in 50 mM sodium acetate buffer, pH 5.0, at 37°C. Three microliters of the enzyme solution (0.5 μM) was added to 100 μL of the substrate solution (10 mM). A portion of the reaction mixture was applied to a gel-filtration HPLC (TSK-GEL G2000PW), and eluted with 0.1 M NaCl with a flow rate of 0.3 mol/min.

HPLC profiles are shown in Figure 23.28. The initial substrate $(GlcN)_3$–GlcNAc was first degraded into $(GlcN)_2$–GlcNAc, and then into GlcN–GlcNAc, but no *N*-acetylglucosamine monomer was produced at this stage (Figure 23.28a). This indicates that the enzyme hydrolyzes the substrate from the nonreducing end in an exo-splitting manner. After a longer incubation period (Figure 23.28b), the substrate was finally hydrolyzed into monosaccharides (GlcN and GlcNAc). It should be noted that transglycosylation products with molecular weights larger than that of the initial substrate were significantly detected (Figure 23.28a). The enzyme might be applicable to enzymatic synthesis of novel oligosaccharides containing a GlcN residue. Similar HPLC analysis was conducted using a mono-*N*-acetylated chitosan dimer (data not shown). The rate of the enzymatic hydrolysis of GlcN–GlcNAc was found to be similar to that of $(GlcN)_2$. This analysis provided information on the specificity of the +1 subsite; i.e., the subsite does not have a strict specificity to the GlcN residue. Thus, the partially *N*-acetylated chitosan oligosaccharides are useful for analyzing the mode of enzyme action and the specificity to GlcNAc or GlcN for individual subsites of chitinases/chitosanases.

23.4.4 Binding Experiments Using Partially *N*-Acetylated Chitosan Trisaccharides

Hen egg white lysozyme has been intensively studied with respect to its structure and function (Fukamizo 2000) and is a model protein for analyzing protein–oligosaccharide interaction. NMR spectroscopy has been used to analyze the interaction of lysozyme with three types of partially *N*-acetylated chitosan trisaccharides (GlcNAc–GlcNAc–GlcNAc, GlcN–GlcNAc–GlcNAc, GlcN–GlcN–GlcNAc). ^{1}H NMR signals derived from several amino acids located inside the hydrophobic core region of lysozyme are quite responsive to saccharide binding (Fukamizo et al. 1992). A typical example of the spectral changes in response to saccharide binding is shown in Figure 23.29. The C5H signal of Trp28 has clearly shifted to the higher field region, and the signal responses were used to determine the association constants of the trisaccharides. The magnitudes of the signal responses were plotted against the free saccharide concentrations to obtain titration curves (Figure 23.30). The association constants were calculated to be 7.1×10^4 M^{-1} for GlcNAc–GlcNAc–GlcNAc, 5.3×10^3 M^{-1} for GlcN–GlcNAc–GlcNAc, and 9.4×10^2 M^{-1} for GlcN–GlcN–GlcNAc, based on the

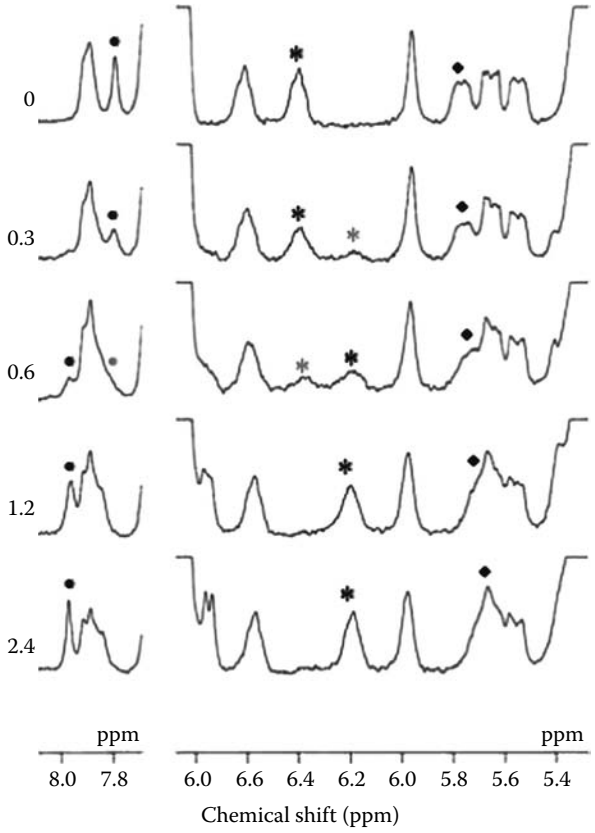

FIGURE 23.29 ^{1}H-NMR spectra showing the $(GlcNAc)_3$ binding to hen egg white lysozyme. The binding experiment was performed in 0.01 M deuterated sodium acetate pH 4.5 at 35°C. The lysozyme concentration was 0.63 mM. Molar ratios of the trisaccharide to the lysozyme are indicated on the left of individual spectra. The resonances of Trp63 C2H (●), Trp28 C5H (*), and Asn59 αCH' (♦) are designated, respectively.

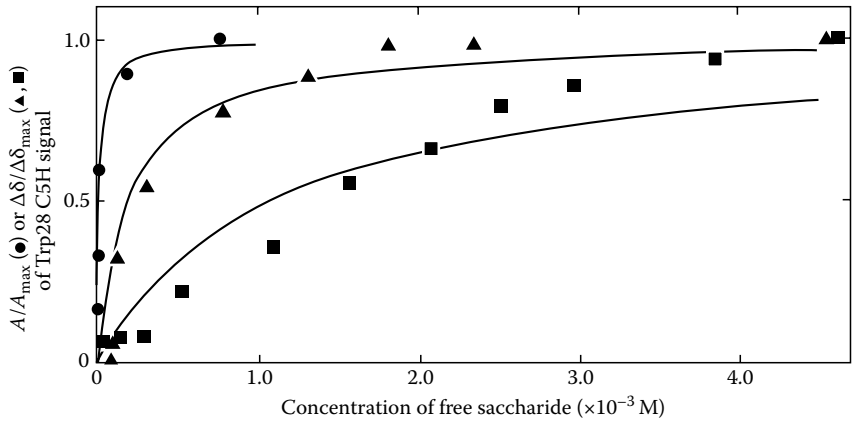

FIGURE 23.30 Titration curves of trisaccharide binding determined from the change in the Trp28 C5H signal. ●, $(GlcNAc)_3$; ▲, GlcN–GlcNAc–GlcNAc; ■, GlcN–GlcN–GlcNAc. Solid lines indicate the theoretical binding curves best fitted to the experimental points.

titration curves. The binding abilities were considerably impaired by removal of the *N*-acetyl group, suggesting the importance of the *N*-acetyl group in the protein–oligosaccharide interaction.

23.5 CONCLUDING REMARKS

Chitin and chitosan oligosaccharides have been used in various aspects of enzymatic analysis of chitinases/chitosanases. Due to their high solubility and homogeneity, various analytical methods including HPLC, NMR, fluorescence, CD, SPR, and ESI-TOF-MS have been successfully applied to hydrolytic and binding experiments for chitinases/chitosanases, and have enabled the determination of the specific activity, kinetic constants, association constants, and subsite affinities of the enzymes toward oligosaccharide substrates. At present, $(GlcN)_n$ and $(GlcNAc)_n$ ($n = 2–6$) are available from commercial source, or can be easily prepared by the methods described in the previous sections. However, it is quite difficult to obtain the oligosaccharides with chain lengths of more than seven units in sufficient purity. In fact, experimental data for enzymatic reactions toward the longer chain length oligosaccharides have not been reported yet.

The oligosaccharides with the chain lengths of 2–6 units might be pseudosubstrates for the polysaccharide-active enzymes, such as chitinases and chitosanases. Thus, some important aspects of the enzymatic reaction toward polysaccharide substrates might be missing in the reactions toward pseudosubstrate oligosaccharides. One of these important aspects is "processivity," in which the single-carbohydrate chains are threaded through the active-site cleft, while terminal disaccharide moieties are cleaved off at the catalytic center (Horn et al. 2006a,b; Zakariassen et al. 2009). This mechanism has been recognized to take place during enzymatic degradation of crystalline polysaccharides, such as cellulose and chitin, and considered to play an important role in polysaccharide degradation. However, such a mechanism might be suppressed in the enzymatic hydrolysis of oligosaccharide substrates, because "processivity" is considered to take place through the cooperative action of a number of contact points between the enzyme-binding cleft and the polysaccharide chain (Sikorski et al. 2006).

Another problem in enzymatic analysis using oligosaccharide substrates is substrate inhibition, which is often observed in the reactions of polysaccharide-active enzymes and interferes with the correct determination of kinetic constants. The long-extended binding cleft of the enzymes can accommodate two oligosaccharide molecules simultaneously, resulting in a dead-end enzyme–substrate complex. This situation might bring about the substrate inhibition phenomena observed in chitinase/chitosanase reactions toward oligosaccharide substrates. In human macrophage chitinase, the substrate inhibition was found to be due to transglycosylation reaction (Aguilera et al. 2003), which is often observed in the reactions of retaining chitinases/chitosanases toward oligosaccharide substrates.

The hydrolytic and binding experiments using oligosaccharide substrates have significantly contributed to the understanding of the structure–function relationship of chitinases/chitosanases. However, enzymatic reactions toward oligosaccharide substrates with the chain lengths of 2–6 units do not always reflect correctly the reaction toward polysaccharide substrates. Since an oligosaccharide is only a part of the corresponding polysaccharide, the enzymatic reaction toward an oligosaccharide substrate might be only a part of the enzymatic hydrolysis toward the entire polysaccharide chain. So the experimental data obtained from the oligosaccharide experiments should be carefully discussed considering the negative aspects described above. Nevertheless, we believe that new analytical techniques will allow us to quantitatively analyze the enzymatic reaction toward oligosaccharide substrates with higher polymerization degree (more than 7 units). The use of such oligosaccharide substrates will remove the negative aspects described above, and bring about a more reasonable and sophisticated model of the polysaccharide degradation.

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24 Low Molecular Weight Water-Soluble Chitosan with Free Amine Group for Drug Delivery

Mi-Kyeong Jang and Jae-Woon Nah

CONTENTS

24.1 INTRODUCTION

The therapy for human diseases using biomedical polymer has evoked a greater interest among researchers in bioengineering fields for its capacity for easy modifications, low cytotoxicity, and wide variety. Especially, core–shell type polymeric micelles have been extensively investigated for drug delivery applications (Kataoka et al. 2001, Nishiyama and Kataoka 2003, Kim et al. 2007a,b). Since polymeric micelles are composed of hydrophobic inner core and hydrophilic outershell, they offer several advantages for drug delivery applications, including hydrophobic cores as a microreservoir of hydrophobic drugs, hydrophilic outershell as a defense layer against attack of the reticuloendothelial system (RES), long blood circulation of drug, and passive targeting against tumor (Thunemann et al. 2000, Kim et al. 2007a,b).

To date, polymeric micelles for the formulation of drug delivery have been frequently prepared using such materials as poly(lactic-*co*-glycolic acid) (PLGA) (Suh et al. 1998), poly(lactic acid) (Feng and Huang 2001), poly(ethylene oxide-*co*-poly(lactic acid)) (Jaeghere et al. 1999), poly(epsilon caprolactone) (Kim and Lee 2001), and poly(propylene oxide) (Lee et al. 2002). These polymeric nanoparticles offer a number of advantages for drug delivery to specific tissue such as tumor; an enhanced loading efficiency of drug by the hydrophobic core, prevention of burst effects, easy modifications of targeting ligands for site specificity, and easy targeting at the desired site by ligand. In spite of these advantages, the synthetic polymers have numerous obstacles such as the complexity of preparation processes, the remnants of surfactants used for preparation of nanoparticle, and their own cytotoxicity in humans.

In view of these limitations, chitosan as a natural polymer has many advantages owing to its biodegradability, biocompatibility, and nontoxicity. Low-molecular-weight water-soluble chitosan (LMWSC) (Jang and Nah 2002) prepared by our laboratory is capable of overcoming the general limitations of chitosan, such as poor solubility in distilled water and the limitation of amine group attributed to its modification for enhanced water solubility. Therefore, in this book, we will focus on issues related to applications and characterization of chitosan as a biomedical polymer for drug or gene delivery. We will also cover chemical modifications of LMWSC using various functional groups to enhance its bioavailability and emphasize its potential as a drug delivery system (DDS).

24.2 CHARACTERISTICS OF LMWSC WITH FREE AMINE GROUP

Chitosan is a biocompatible, biodegradable, nontoxic, and cationic polymer. Due to its amino functional groups, chitosan has been extensively investigated in the areas of drug delivery, gene delivery, and biomedicine (Hirano 1999, Thanou et al. 2001, Dang and Leong 2006), and has been reported to enhance drug delivery across the nasal or mucosal layer without damage (Chae et al. 2005a,b). Especially, the cationic properties of chitosan offer valuable properties for drug delivery systems and gene delivery systems. For instance, ion complex formations between chitosan and anionic drug or DNA are available, and these kinds of ion complexes can be prepared simply by mixing into aqueous solutions (Calvo et al. 1997, Chae et al. 2005a,b, Kim et al. 2006).

Especially, water-soluble chitosan (WSC) provides remarkable advantages when compared with water-insoluble chitosan (Ouchi et al. 1998). Water-insoluble chitosan normally requires use of acidic solution to improve its aqueous solubility. However, this problem is critical for application of DNA, protein or peptide drug, and anticancer drugs because these bioactive agents are unstable in acidic solution. An enhanced aqueous solubility of chitosan promises distinguished potential in the biomedical field. To enhance water solubility of chitosan, various ideas have been attempted such as PEGylation (Jeong et al. 2008), carboxymethylation (Muzzarelli et al. 1982, Chen et al. 2004), and decrease of molecular weight (Jang et al. 2002).

However, it is not easy to chemically modify chitosan bound to water-soluble group for the purpose of introducing the functional group, which can be specified in the target tissue because of the decrease in the reactivity or positive charge of amine group. In our previous study (Jang and Nah 2002), low-molecular-weight water-soluble chitosan (LMWSC) distinguished from other kinds of chitosan was prepared by the salt removal method (Figure 24.1). Various salts binding to the amine group of chitosan can be removed by this method, as shown in Figure 24.2. Especially, it should be noted that the salt removed LMWSC has enhanced bioactive properties such as enhanced immunoactivity, antibacterial activity, and enhanced drug incorporation.

Gel permission chromatograpy (GPC) and ^{1}H NMR spectra were used to characterize LMWSC with free-amine group, LMWSC and chitosan with lactic acid as a salt (COS) in Figures 24.3 and 24.4 (Jang et al. 2007), respectively. GPC was measured to confirm the molecular weight of LMWSC, and also retention time was compared to COS according to molecular weight. It can be

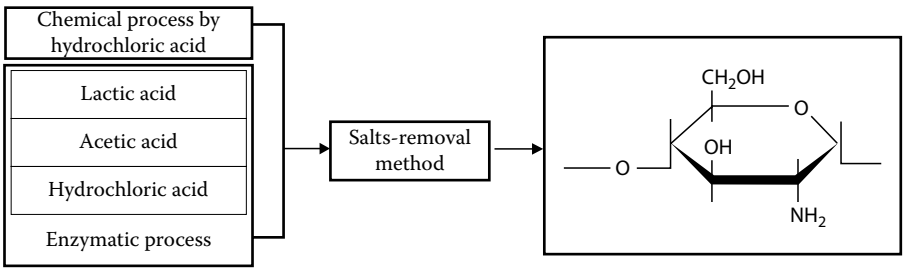

FIGURE 24.1 The scheme of salts-removal method for LMWSC.

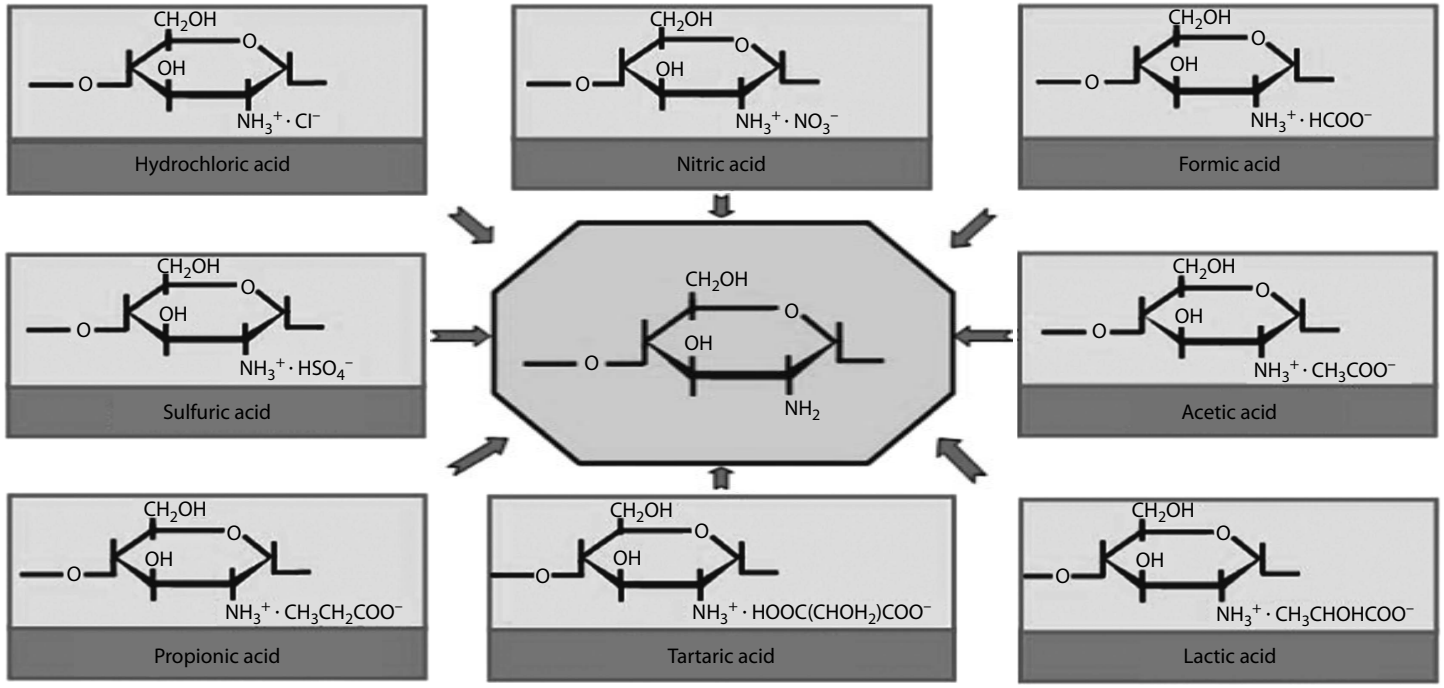

FIGURE 24.2 Various amine salts prepared by acidic decomposition of chitosan.

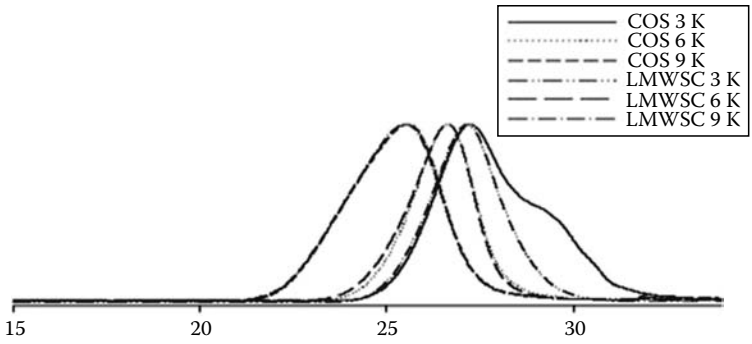

FIGURE 24.3 Gel permeation chromatograph of COS and LMWSC. (From Jang, M.J. et al., *Polymer* (*Korea*), 36, 555, 2007. With permission.)

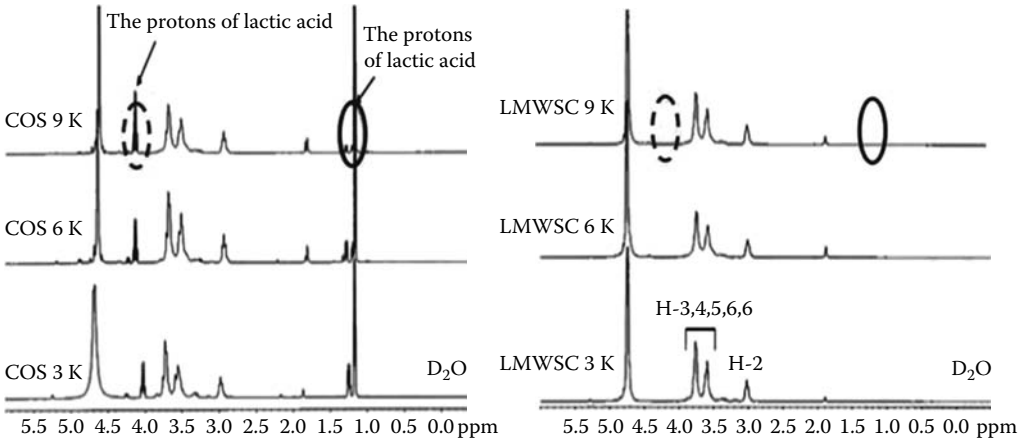

FIGURE 24.4 ^{1}H-NMR spectra of COS and LMWSC. (From Jang, M.J. et al., *Polymer* (*Korea*), 36, 555, 2007. With permission.)

seen in Figure 24.3 that molecular weights of LMWSC are 3, 6, and 9 KDa, respectively. It was similar to the molecular weight of COS but the retention time of LMWSC was a little fast compared to COS because of the removal of lactic acid. The PDI value of LMWSC showed a unimodal molecular weight distribution from 1.279 to 1.499.

Figure 24.4 shows that the peak between 1 and 2 ppm by lactic acid of COS has disappeared in the cases of molecular weights of 3,000, 6,000 and 9,000 Da (3, 6, and 9 kDa. This means that LMWSC with free amine was successfully prepared because the lactic acid linked at amine group of COS was removed by the salt removal method (Jang and Nah 2002). These findings led us to expect that LMWSC removed salts have superior potential to be applied to drug or gene delivery by introducing various functional groups through chemical modification and also to expect that it is possible to target at the specific tissue.

24.3 MODIFICATION OF LMWSC WITH FREE AMINE GROUP AS A DRUG CARRIER

Nanoparticles as a drug carrier based on biodegradable polymers are also a useful device for intravenous administration of hydrophobic drug to the body because of sustained drug action on the lesion, reduced systemic side effects, high capability to cross various physiological barriers, especially facile extravasation into tumor, as well as controlled and targeted delivery (Desai et al. 1997,

Feng et al. 2000, Brigger et al. 2002). Among the various biopolymers for preparation of nanoparticle, chitosan modified with a functional group has been extensively investigated as biomaterials and carriers of hydrophobic drug. Especially, LMWSC having a free-amine group can be modified by various functional groups, which can target in the site desired for enhanced drug delivery efficiency.

24.3.1 The Formation of Complex by Ionic Interaction

Devices for drug delivery are very important not only for targeting drug to the desired site of action, but also for prolonged blood circulation of drug, as well as for reduced side effects of hydrophobic drug. Especially, as one of the various devices, nanoparticles have been extensively studied in drug delivery systems for drug targeting (Davis et al. 1993). Due to their reduced particle size, nanoparticles are susceptible for intravenous (i.v.) injection. As a model drug for application of nanoparticle, all *trans*-retinoic acid (ATRA) has been reported to be effective in the treatment of epithelial and hematological malignancies such as breast cancer (Kalmekerian et al. 1994), human malignant gliomas (Defer et al. 1997), head and neck cancer (Giannini et al. 1997), ovarian adenocarcinoma (Krupitza et al. 1995), and acute promyelocytic leukemia (APL) (Huang et al. 1988). Since R–COOH group has a negative charge, a complex can be formed by ionic interaction between RA and LMWS, which have many positive charges.

LMWSC nanoparticles based on polyelectrolyte complexes with at RA can be prepared by mixing atRA (dissolved in DMF) into the chitosan aqueous solution with an ultrasonication followed by the dialysis of mixed solution. Nanoparticles based on polyelectrolyte complexes between LMWSC and atRA must be formed through ion complex formation between the amine group of chitosan and the carboxyl group of atRA as shown in Figure 24.5 (Kim et al. 2007a,b) These LMWSC complexes show high drug loading efficiencies with a small particle size of below 400 nm. Most of the other reports have shown that the particle size increased with increasing atRA loading contents (Ezpeleta et al. 1996, Thunemann and Beyermann 2000, Thunemann et al. 2000, Lim et al. 2002). Choi et al. (2001) have reported that a high efficiency of drug loading over 90% (w/w) is obtained in the microspheres, but their

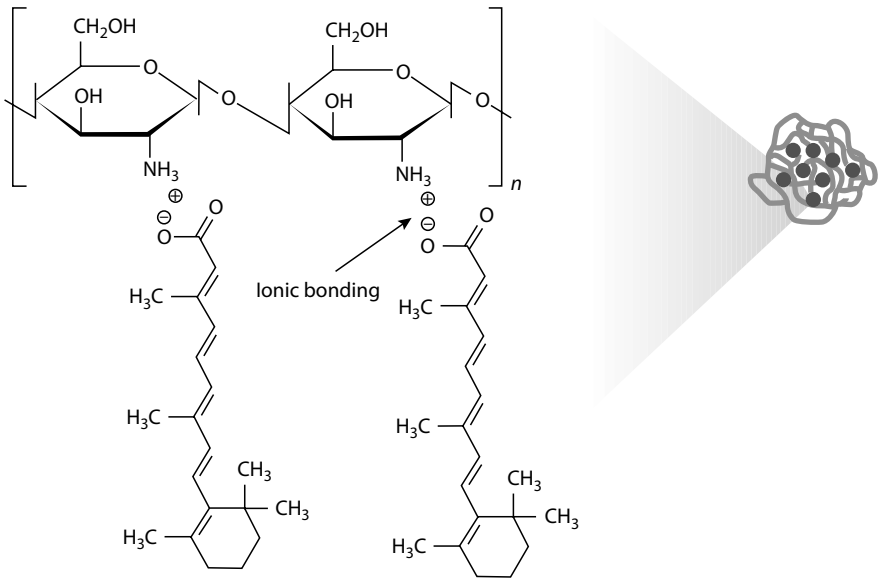

FIGURE 24.5 Structural scheme of ion complex formation between chitosan and atRA. (From Kim, D.G. et al., *J. Appl. Polym. Sci*., 105, 3246, 2007a; *Key Eng. Mater*., 342, 469, 2007b. With permission.)

particles have several micrometers in particle size, which are not acceptable for intravenous injection. Gliadin nanoparticles also have a high entrapment efficiency of about 75% (w/w), but their particle sizes are higher than 500 nm (Ezpeleta et al. 1996). Lim and Kim (2002) have reported atRA-entrapped solid lipid nanoparticles of below 200 nm in particle size, which have very low drug contents.

24.3.2 PEGylation for Delivery of Hydrophobic Drug

PEGylation as a hydrophilic group has advantages in drug delivery, such as prevention of cell adhesion by entropically driving steric repulsion and by increasing the hydrophilicity of carrier surfaces. We have investigated its potential for the delivery of methotrexate using ChitoPEG (Jeong et al. 2009). Methotrexate is widely used in the treatment of various malignancies including childhood acute lymphocytic leukemia, osteosarcoma, non-Hodgkin's lymphoma, Hodgkin's disease, head and neck cancer, lung cancer, breast cancer, psoriasis, choriocarcinoma, and related trophoblastic tumors (Calabresi and Parks 1975). However, undesirable side-effects of MTX has been reported such as toxic side-effects to normal cells, drug resistance, nephrotoxicity, bone marrow suppression, acute and chronic hepatotoxicity, interstitial pneumonitis, and chronic interstitial obstructive pulmonary disease (Calabresi and Chabner 1999). Various delivery systems for antitumor therapy using MTX have been proposed to solve this problem.

Figure 24.6 (Jeong et al. 2009) shows the scheme of MTX-incorporated polymeric micelle using MPEG-grafted LMWSC (abbreviated as ChitoPEG) to develop an antitumor drug delivery system. ChitoPEG can be easily synthesized as reported (Kim et al. 2007a,b) previously by using LMWSC that has a free amine group. Size distribution of MTX-incorporated polymeric micelles is narrowly distributed, and the average particle size is below 100 nm, which is suitable for drug delivery. The release of MTX from polymeric micelles of ChitoPEG copolymer was sustained for 4 days and decreased with decreasing drug contents. Also, the higher DS of MPEG induced an increased drug release rate from the polymeric micelles (Figure 24.7), indicating that polymeric micelles of ChitoPEG copolymer can be considered for a controlled release vehicle of MTX (Jeong et al. 2009).

In other applications of ChitoPEG, the carrier for anticancer agent has been characterized by modifying the hydrophobic moiety. Paclitaxel as a typical hydrophobic anticancer agent has been known to have a significant activity to a wide range of tumors, but it has a serious problem including toxic side effects and poor solubility in the conventional aqueous injection solution. Especially,

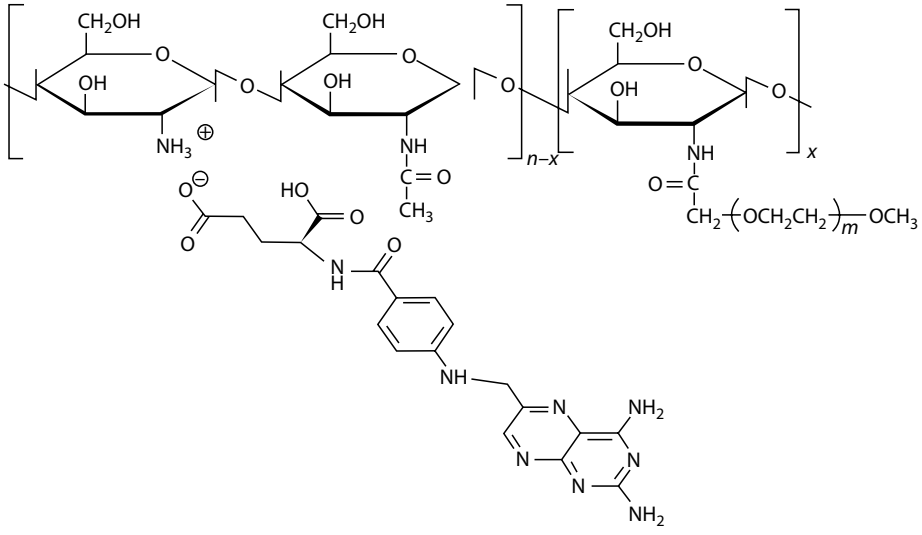

FIGURE 24.6 Scheme of polymeric micelle formation between MTX and ChitoPEG copolymer.

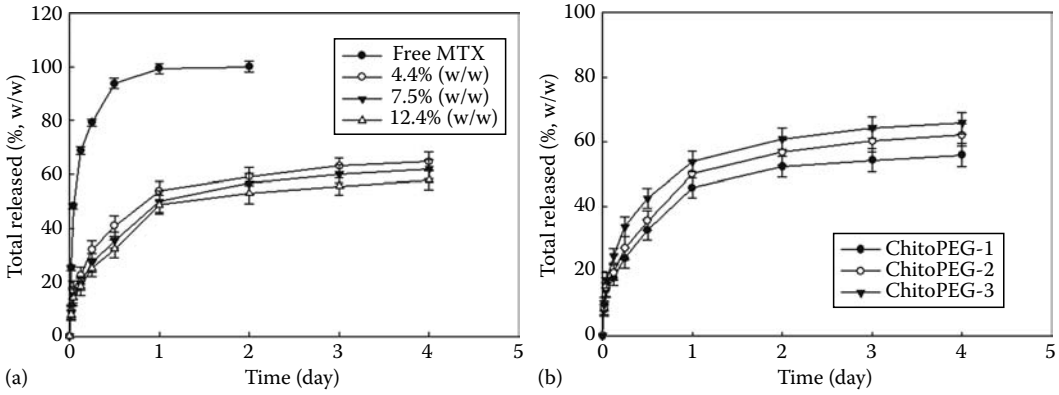

FIGURE 24.7 Drug release from polymeric micelle of ChitoPEG copolymer. The effect of drug contents (a) and M.W. of ChitoPEG copolymer (b); DS of ChitoPEG-1, 2, 3:2.5, 5.15, 11.5, respectively. (From Jeong, Y.I. et al., *Macromol. Res.*, 17, 538, 2009. With permission.)

low therapeutic index of paclitaxel is known to cause toxic side-effects (Weiss et al. 1990). Because of the low solubility of paclitaxel (Liggins et al. 1997), it has to be dispersed in a mixed solution of cremophore EL (polyethoxylated oil) and ethanol (50:50), which is diluted with 5- to 20-fold in normal saline or 5% dextrose solution for intravenous injection. Approaching the problem from this point of view, various systems have been proposed to make paclitaxel formulations for injection such as parenteral emulsion (Tarr et al. 1987, Lundberg 1997), mixed micelles (Greenwald et al. 1996), water-soluble prodrugs (Zhang et al. 1996), polymer micelles (Yokoyama et al. 1990), core–shell-type nanoparticles (Jeong et al. 2005), albumin-bound nanoparticles (Harries et al. 2005), and biodegradable polymeric nanoparticles (Gaucher et al. 2007).

For this formulation of paclitaxel, ChitoPEG modified with hydrophobic group has been prepared by the previous method (Jang et al. 2002). The introduction of cholesterol as a hydrophobic group can enhance the association behavior of LMWSC, and the stability and activity of the hydrophobic drug can be enhanced by formation of hydrophobic core. Core–shell-type nanoparticles composed of ChitoPEG and cholesterol (ChitoPEG–Chol) have a unique chemical structure as shown in Figure 24.8a, and the core–shell structure should be formed in the aqueous environment as shown in Figure 24.8b. For instance, cholesterol conjugated in the chitosan main chain may form a hydrophobic core of the core–shell-type nanoparticles and PEG may be formed in the hydrated outershell.

The antitumor efficacy of ChitoPEG–Chol nanoparticles is most effective in suppressing tumor growth with a high dose of paclitaxel while paclitaxel at equivalent dose is less effective to suppress tumor growth (data not shown) (Nah et al. 2004). Although tumor growth in both paclitaxel and core–shell-type nanoparticles treatment at a low dose of paclitaxel was not significantly different, the core–shell-type nanoparticles at a high dose of paclitaxel has recorded a higher survival ratio until 50 days. Furthermore, body weight changes were also smaller than that in paclitaxel treatment at high dose. These results indicate that the core–shell-type nanoparticles of ChitoPEG–Chol are suitable paclitaxel carriers for inhibition of tumor growth at in vivo with minimal side-effects. This means that the enhanced antitumor activity may be attributed to the fact that the core–shell-type nanoparticles prepared by ChitoPEG–Chol have effective targeting properties to tumor while paclitaxel itself has no selectivity.

24.3.3 LMWSC and Its Derivatives for DNA Delivery

In a similar approach to drug delivery, gene delivery provides important opportunities to treat various kinds of life-threatening and gene-related diseases by producing biologically active agents or stopping abnormal functions of the cells such as genetic failure or uncontrollable proliferation of cells. Polycationic polymer as a gene delivery can overcome the dangerous problem inducible

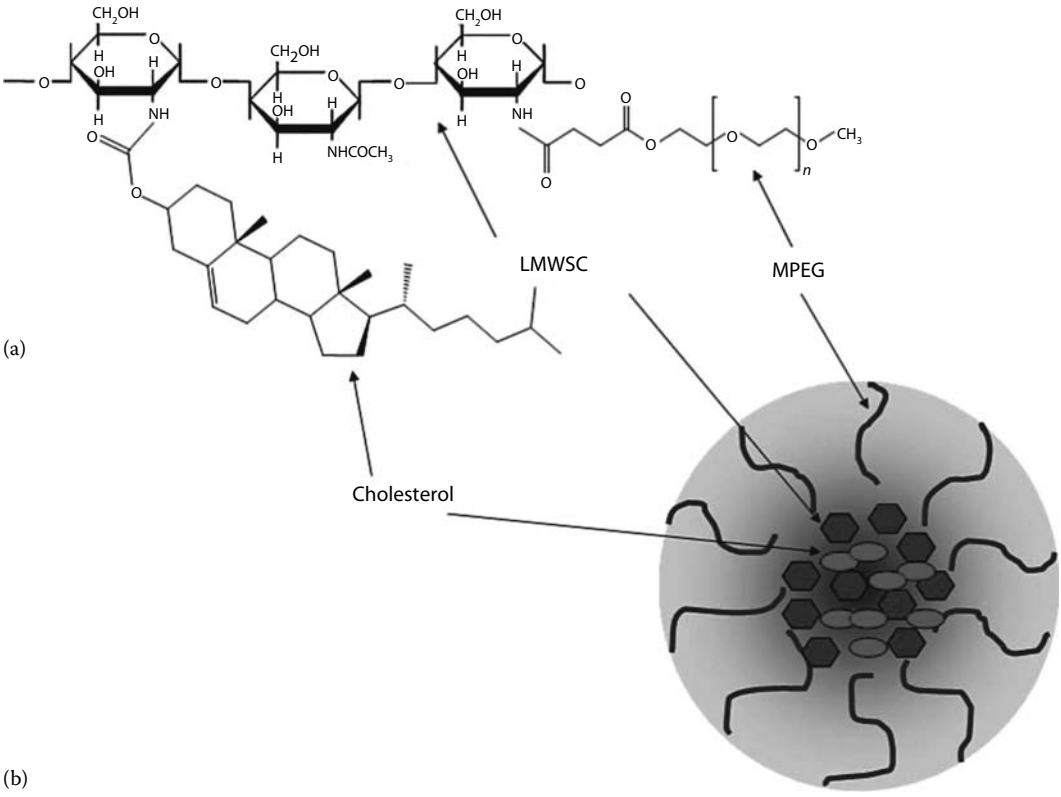

FIGURE 24.8 (a) Chemical structure of ChitoPEG-Chol and (b) schematic illustration of core-shell type nanoparticles.

in the human unlikely viral vector. Cationic polymers including poly(L-lysine) (PLL) (Wu and Wu 1987, 1998) and polyethylenimine (PEI) are able to condense plasmid DNA and protect it from enzymatic degradation, which results in the enhancement of the transfection efficiency. However, there still remain such drawbacks as biocompatibility and cytotoxicity in the body. To overcome the biocompatibility problem, nontoxic biodegradable polymeric gene carrier has been developed as a promising gene delivery material (Behr 1993).

In this approach, LMWSC with free amine group has many advantages to deliver safety gene in the body. The amine group of glucosamine unit in the structure of chitosan has several advantages as a gene carrier such as noncytotoxicity and strong positive charge, which facilitates formation of the complex with DNA. The transfection efficiency of LMWSC is increased with increasing molecular weight due to high positive charge (Figure 24.9) (Jang et al. 2007).

In another study of enhanced gene delivery efficiency, we prepared a gene carrier with the same design as the ChitoPEG–Chol described above and tested the potential for gene delivery. This carrier can be specified at the tissue desired by the LDL receptor mediated endocytosis process because of the cholesterol modified as a hydrophobic moiety. Thus it can be used to obtain an enhanced expression efficiency (Figure 24.10) (Choi et al. 2006). In this study, the transfection efficiency was investigated in HCT 116 cell lines using pEGFP-N1 plasmid encoding green fluorescence protein (GFP). The gene expression was observed by the confocal microscopy. As shown in Figure 24.10, the transfection efficiency of complexes is enhanced by introducing MPEG and cholesterol on the LMWSC. The transfection efficiency of the LCP modified with MPEG was twice as much as that of LMWSC while the LCP-Ch enhanced the transfection efficiency 50 times more than LMWSC. This result indicates that the gene transfection efficiency can be enhanced by facilitating the cellular

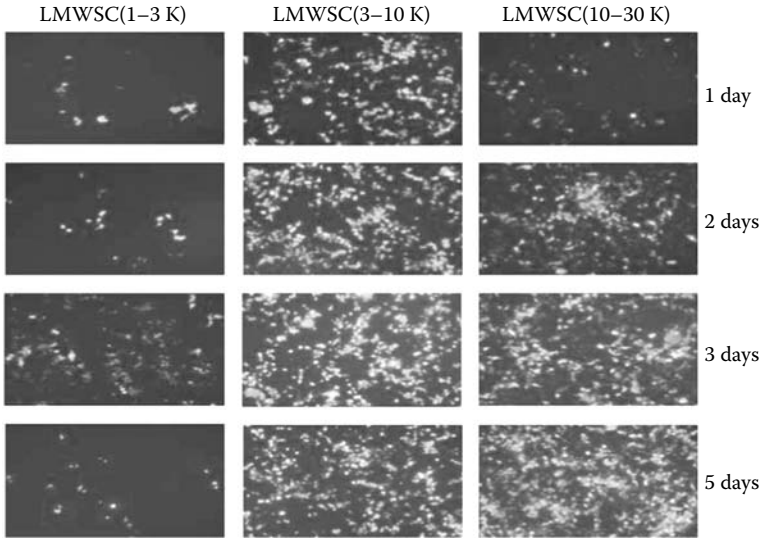

FIGURE 24.9 The effect of transfection efficiency of DNA according to molecular weight of LMWSC. (From Jang, M.J. et al., *Polymer* (*Korea*), 36, 555, 2007. With permission.)

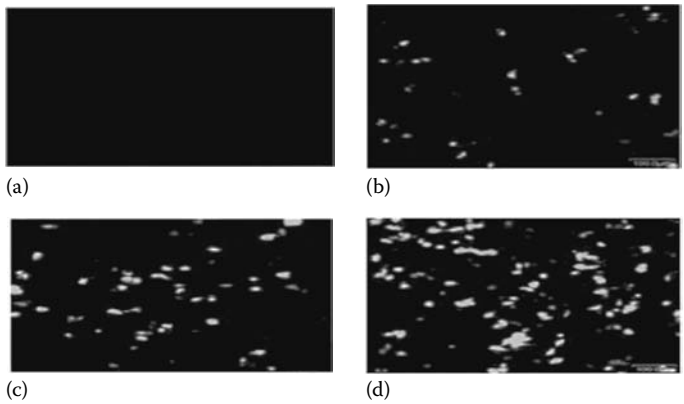

FIGURE 24.10 Transfection efficiency of LMWSC derivative. (From Choi, C. et al., *J. Appl. Polym. Sci*., 102, 3545, 2006. With permission.)

uptake of the carrier/DNA complex that introduces the hydrophobic and hydrophilic moieties. In the case of LMWSC itself as presented above in Figure 24.9, its transfection efficiency is higher than modified LMWSC with PEG and Chol. It seems that the positive charge of LMWSC was decreased by the modification at the free amine group. Thus, polycationic material needs to be modified to enhance the efficiency.

24.4 TARGETING BY ENDOCYTOSIS PATHWAY-MEDIATED LDL RECEPTOR USING LMWSC DERIVATIVE

To date, systemic chemotherapy for cancer has had major obstacles such as resistance to cytotoxic drug, excruciating pain, poor solubility of hydrophobic anticancer agent in distilled water, and cytotoxicity in the body (Dhanikula and Panchagnula 1999). Moreover, reduced intracellular drug accumulation in resistant cells may ascribe to an inhibition of drug uptake and an increase in drug

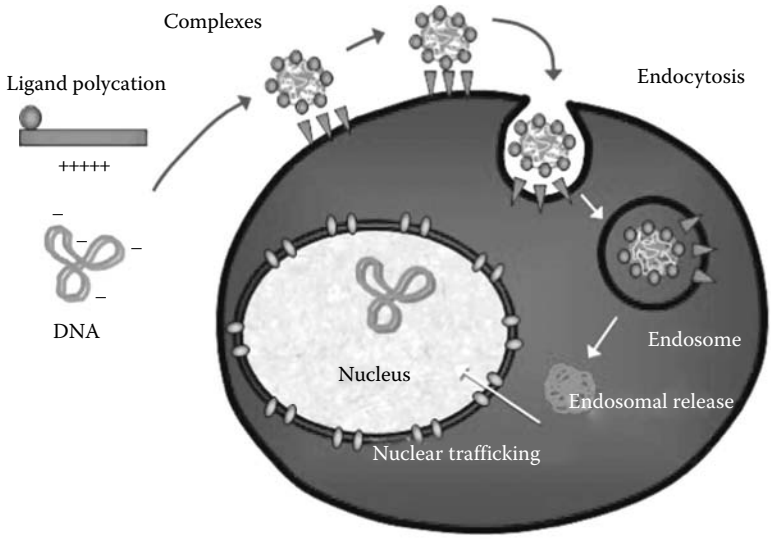

FIGURE 24.11 Receptor mediated endocytosis process.

efflux. To overcome these problems, development of a vehicle for efficient therapy of cancer has been called for by investigators. Among the various factors, the tumor targeting using a drug carrier is most important in reducing the side effect in the case of systemic delivery of anticancer agent. Tumor cell targeting is a promising strategy for enhancing the therapeutic potential of chemotherapy agents (Wu et al. 2006) using the receptor-mediated endocytosis mechanism (Figure 24.11).

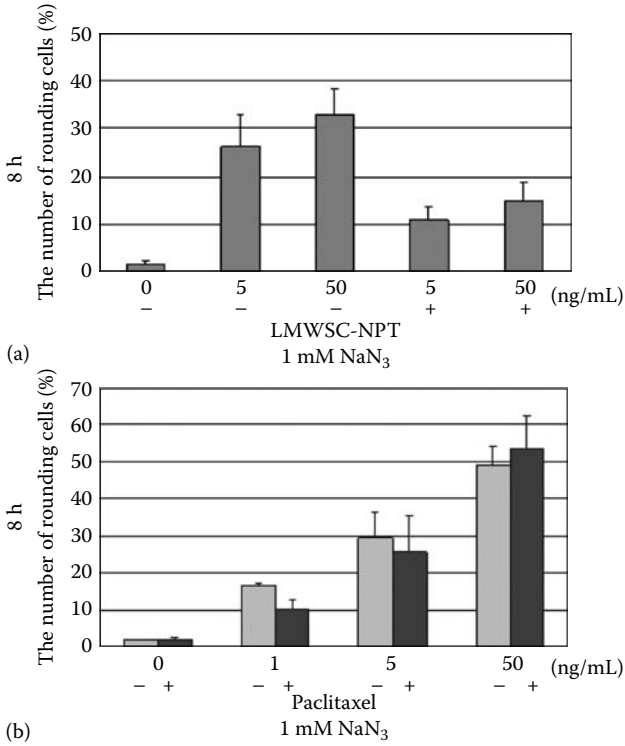

FIGURE 24.12 Anticancer activity of LMWSC-NPT (a) and paclitaxel (b) according to the effect of inhibitor. (From Kim, D.G. et al., *J. Appl. Polym. Sci*., 105, 3246, 2007a; *Key Eng. Mater.*, 342, 469, 2007b. With permission.)

It is well known that LDL can be taken up by different types of cells by the LDL receptor existing on the cell surface via receptor-mediated endocytosis (Nah et al. 2002). ChitoPEG–Chol with paclitaxel (LMWSC nanopaclitaxel, LMWSC-NPT), as described above, is used to confirm this mechanism. Using the sodium azid as an endocytosis inhibitor, the anticancer activity of ChitoPEG with cholesterol, which is the ligand into cancer cell as a carrier of paclitaxel was compared to that of control paclitaxel to identify the receptor-mediated endocytosis mechanism by LDL receptor. Figure 24.12a (Kim et al. 2007a,b) shows that when sodium azid does not exist, the anticancer activity increases with increasing LMWSC-NPT concentration. On the other hand, the activity of LMWSC-NPT with sodium azid decreased. It seems that sodium azid inhibits endocytosis pathway by the receptor. The anticancer activity of paclitaxel as a control does not change whether the sodium azid as an inhibitor exists or not (Figure 24.12b). These findings reveal that the anticancer activity of LMWSC-NPT differed from paclitaxel as a control depending on the existence of the inhibitor. This result indicates that the antitumor activity of LMWSC-NPT that introduced cholesterol as an LDL receptor is much higher than that of control paclitaxel because of the endocytosis process by the LDL receptor.

24.5 CONCLUSIONS

For safe delivery drug in the human body, a suitable device was needed such as nanoparticle, microcapsule, hydrogel, and other forms of dose. Especially, nanoparicles offer important advantages such as targeted drug delivery to the desired site of action, prolonged blood circulation of drug, and reduced side effects of anticancer drug. Such applications of nanoparticles on the drug targeting to the specific body sites have great advantages in avoiding the surgery, which can always be the source of infection. As pointed out in this review, nanoparticles can be prepared simply by ionic interaction and modification with hydrophilic and hydrophobic groups using LMWSC. Also, LMWSC and its derivatives seem to be good devices for delivery of bioactive materials. However, more detailed in vivo study needs to be carried out for clinical trials since the majority of the studies reported to date are in vitro and in animal models.

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25 Chitosan/Chitosan Derivatives as Carriers and Immunoadjuvants in Vaccine Delivery

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CONTENTS

25.1 INTRODUCTION

Immunization is an approach adopted to provide a protective shield to the body so that it is protected against any incoming pathogenic invasion. To be effective, immunization strategies and schedules are planned to elicit strong and long-lasting immune response at both cellular and humoral levels. In most of the cases, immune response is generated by administering an antigen in association with an adjuvant for producing efficient immunological consequences. Polymers, lipids, and inorganic compounds such as alum and their derived forms, which are known as vaccine delivery systems, have been developed for generating an adjuvant-like effect.

Chitosan is a biocompatible biomaterial with a multitude of applications particularly in the fields of biomedical engineering and drug/vaccine delivery. Prophylactic protection through strong immunity against an infectious disease can be achieved only with vaccines that do not require frequent dosing as in the case of conventional drug therapy (Alpar et al. 2005). Systemic and mucosal immunity are both equally needed for effective vaccination. Most of the vaccination strategies suffer from the problem of low antibody titer due to weak immune response. Hence these strategies require the coadministration of either some immunoadjuvants or antigen encapsulated within a carrier system, which may serve as an adjuvant as well as controlled delivery of antigen to the cells involved in immune responses (Anderson 1997; Bacon et al. 2000; Moschos et al. 2004). Chitosan,

a biopolymer, is well known for its pragmatic applications in the field of drug delivery owing to its interesting biological properties including biocompatibility, bioresorbability, and bioactivity (Illum et al. 2001). Since chitosan easily forms microparticles and nanoparticles that can encapsulate a large amount of antigens such as ovalbumin, diphtheria toxoid, or tetanus toxoid, it can be better utilized for controlled vaccine delivery and evoking immunological events including both the development of humoral as well as cellular immunity responses (Jain et al. 2006). Chitosan, being cationic in nature, exhibits advantages as a vaccine carrier due to its intrinsic immune stimulating activity and bioadhesive properties, which further improve the cellular uptake and assist in the permeation of the antigen and also protect antigens both in vitro and in vivo (Marcinkiewicz et al. 1991; Khatri et al. 2008a,b). Similar effects have also been documented for chitosan derivatives (Jiang et al. 2007; Sayin et al. 2008). This is why chitosan and its derivatives are being routinely studied and explored by various scientific groups. Chitosan-based carriers have been developed for noninvasive vaccine delivery (nasal and oral routes). They reportedly showed promising results and their application is projected to be an alternative needle-free vaccination strategy, resulting in more patient compliance. Some of the important chitosan and its derivatives, which have been utilized for vaccine delivery, are shown in Figure 25.1. The chapter discusses the immunological events associated with the use

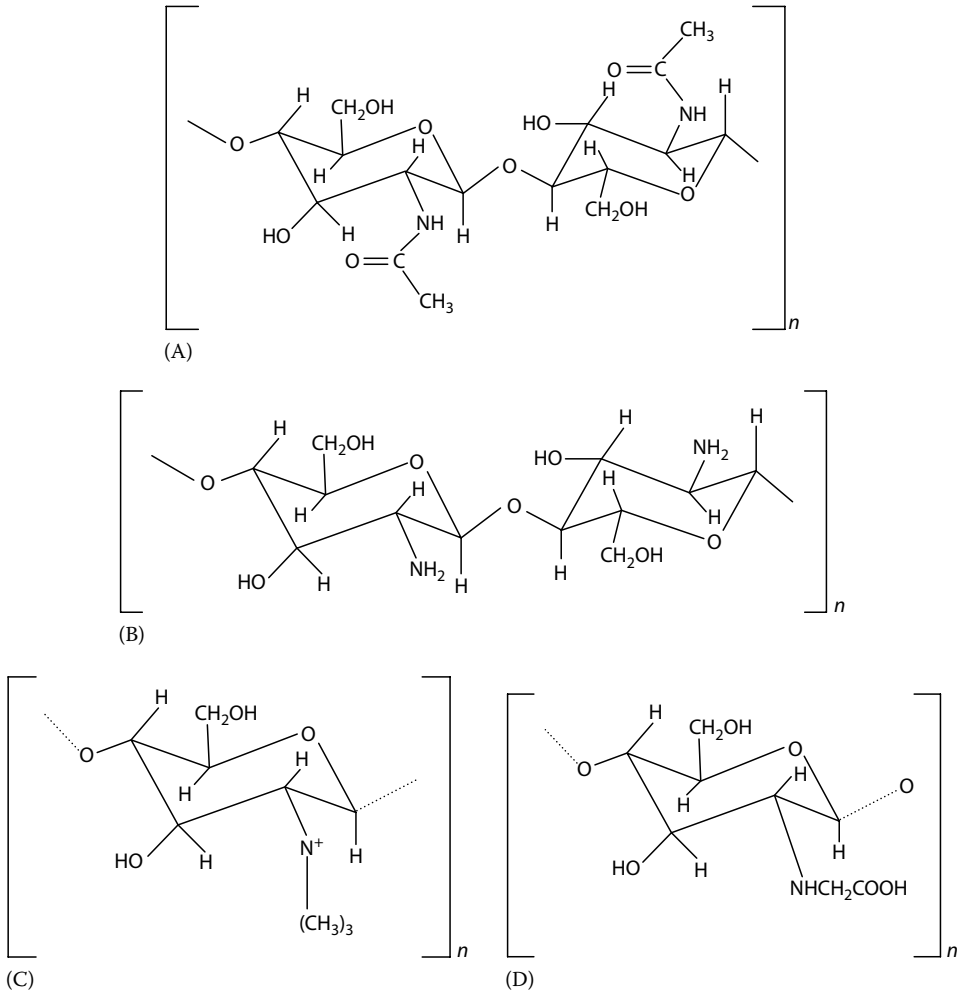

FIGURE 25.1 Chemical structures of chitin and chitosan derivatives which have been utilized as immunoadjuvants (A) chitin; (B) chitosan; (C) trimethyl chitosan; (D) mono-*N*-carboxymethyl chitosan.

of chitosan either as a whole or in part as a carrier system especially designed for vaccine delivery. The evaluation of CS-based micro/nanoparticles as antigen carrier as compared with other system is discussed and presented.

25.2 CHITOSAN AS IMMUNOADJUVANTS: SUITABLE PROPERTIES

Although a number of adjuvants are widely investigated, chitosan is unique among them (Anderson 1997; Bacon et al. 2000). Chitosan, a natural polymer obtained by alkaline deacetylation of chitin, is cheap, nontoxic, biocompatible, and biodegradable. It also serves as an excellent candidate for vaccine delivery because of its bioadhesiveness, high protein-binding/carrying efficiency, and trans biobarrier absorption-enhancement ability (Marcinkiewicz et al. 1991). Due to its polycationic nature, it readily adheres to negatively charged surfaces, such as mucus and proteins, and promotes drug absorption. A large number of studies have demonstrated that chitosan and their derivatives (*N*-trimethyl chitosan, mono-*N*-carboxymethyl chitosan) are effective as safe absorption enhancers, which could be used to improve mucosal (nasal, peroral) delivery of biomacromolecules and vaccine (Nishimura et al. 1984; Xie et al. 2007; Yang et al. 2007; Zhang et al. 2007; Zhou et al 2008). The absorption-enhancing effect of chitosan is attributed to the opening of the intercellular tight junctions by chitosan, thereby favoring the paracellular transport of macromolecular drugs (van der Lubben et al. 2001a,b,c). The nano- and microparticles of chitosan are also suitable biomaterials for amelioration of release profile of drug(s) and vaccine(s). The association of antigen(s) to the particulate systems based on chitosan has shown enhanced uptake of antigen by mucosal lymphoid tissues, thereby inducing strong systemic and mucosal immune responses against the antigens (Bramwell and Perrie 2006). Moreover, the specific adjuvant activity of chitosans relates and depends on the degree of deacetylation and the type of formulation (Nishimura et al. 1984; van der Lubben et al. 2001b). Chitosan, being mucoadhesive in nature, provides prolonged muco/bioadhesion of the nanoparticles and hence longer contact time with blood-supplying capillaries, which results in a higher uptake of the protein antigen/plasmid DNA (Khatri et al. 2008a,b). Opening of tight junctions of the epithelium linings facilitates diffusion of the macromolecules such as antigens through paracellular path. The metabolic intermediates generated on degradation of chtiosan are nontoxic by nature. At mucosal sites, chitosan exhibits immunomodulatory activities and stimulates the release of regulatory cytokines (Baek et al. 2007). This is how chitosan particulate carriers are most attracting scientific endeavor in the formulations and drug delivery research (Table 25.1). Some of the interesting features of chitosan, which make it suitable for vaccine delivery, are shown in Figure 25.2.

25.3 CHITOSAN MICRO/NANOPARTICLES IN IMMUNIZATION

A successful vaccination requires an effective delivery vehicle for protein antigens or plasmid DNA-expressing protein antigen (Bivas-Benita et al. 2003). Chitosan can be suitably manipulated and transformed to particles in the micro- and nanosize range depending on the requirement, route of administration, and the purpose specific delivery of antigen (Panos et al. 2008). Chitosan microspheres are also suitable for controlled release of encapsulated antigen (Jiang et al. 2004). The surface modifications and chemical engineering of carrier is sometimes desired, which is relatively easy in case of chitosan due to active group(s) associated with chitosan molecule. Chitosan, in the form of antigen-loaded particulate carrier, has been explored for vaccine delivery through all major routes of administration including pulmonary, subcutaneous, oral and nasal, etc. (Bivas-Benita et al. 2004; Alpar et al. 2005; Kang et al. 2006; Ahire et al. 2007; Amidi et al. 2007a,b; Khatri et al. 2008a,b). The particulate form of the chitosan makes it suitable for uptake particularly by and through polar epithelial cells to the mucous-associated lymphatic tissues (MALT), which is the major site for antigen processing and hence for architecture of immune response

TABLE 25.1
List of Chitosan-Based Carrier Developed for Vaccine Delivery either Alone or in Combination with Other Materials

Chitosan/Chitosan with Other Material	Carrier	Antigen	Microbe/Disease	Route	Remark	References
Chitosan	Microsphere	Plasmid DNA	Japanese flounder (Paralichthys oilcake's)	Oral	Chitosan microspheres act as promising carriers for oral pDNA vaccine in comparison with fish vaccinated with naked pDNA	Tian et al. (2008)
Chitosan	Nanoparticles	DNA vaccine	*Vibrio* (Listonella) *anguillarum*.	Oral	A relative percent survival (RPS) rate of 46% was recorded. Sea bass (*Lates calcarifer*) orally vaccinated with chitosan–DNA (pVAOMP38) complex showed moderate protection against experimental *V. anguillarum* infection	Rajesh Kumar et al. (2008)
Squalane oil	Chitosan-modified W/O/W Multiple emulsion	Ovalbumin	Immunization potential study	Oral and intranasal	Squalane oil-containing W/O/W multiple emulsion formulations can significantly enhance the local and systemic immune responses, especially after oral administration	Shahiwala and Amiji (2008)
Alginate coated chitosan	Nanoparticles	Recombinant hepatitis B antigen	Hepatitis B	Oral	Coated chitosan nanoparticles might have potential for being used as a deliver system for oral vaccination with the recombinant hepatitis B surface antigen	Borges et al. (2007)
Chitosan	Microparticle	Tetanus toxoid	*Clostridium tetani*	Oral	Improved efficacy of chitosan microparticle suspension system, in inducing the IgA in intestine and IgG in systemic circulation was confirmed	Ahire et al. (2007)
N-trimethyl chitosan	Nanoparticles	FITC-BSA (Model Protein), Urease	Immunization potential study	Oral	Urease loaded TMC nanoparticles showed much higher antibody titers of both IgG and secretory IgA than those with urease solution or urease co-administrated with TMC solution	Chen et al. (2008a,b)
Chitosan within vesicular systems	Nanoparticles within liposome and niosome	BSA	Immunization potential study	Oral	Significantly higher ($P < 0.05$) serum IgG titers were achieved following oral administration of novel nanoparticulate vesicular formulations as compared with unmodified chitosan nanoparticles. Further, high sIgA levels in mucosal secretions advocated a possible application of chitosan nanoparticle encapsulated in vesicles as an oral vaccine delivery carrier-adjuvant system	Jain et al. (2006)

Eudragit coated chitosan (ER-Chi)	Microparticles	Ovalbumin	Immunization potential study	Oral	ER-Chi-OVA may be useful to induce an intestinal mucosal immune response	Hori et al. (2005)
Chitosan	Nanoparticles	Plasmid DNA	House dust mite allergen, Der p 1	Oral	Chitosan–DNA nanoparticles successfully induced oral immune responses specific to both the left and right domains of Der p 1.	Chew et al. (2003)
Chitosan	Microparticles	Diphtheria toxoid	*Corynebacterium diphtheriae*	Oral and Nasal	DT associated to chitosan microparticles results in protective systemic and local immune response against DT after oral vaccination, and in significant enhancement of IgG production after nasal administration	van der Lubben et al. (2003)
Chitosan	Nanoparticles	DNA (pCMVArah2)	Peanut allergy	Oral	Oral allergen-gene immunization with chitosan–DNA nanoparticles is effective in modulating murine anaphylactic responses, and indicated its prophylactic utility in treating food allergy	Roy et al. (1999)
Low molecular weight chitosan	Nanoparticles	Tetanus toxoid	*Clostridium tetani*	Nasal	Nanoparticles made of low Mw CS act as promising carriers for nasal vaccine delivery	Vila et al. (2004)
Chitosan	Nanoparticles	Plasmid DNA	Hepatitis B	Nasal	The study signifies the potential of chitosan nanoparticles as DNA vaccine carrier and adjuvant for effective immunization through non-invasive nasal route	Khatri et al. (2008b)
Chitosan	Chtiosan + inactivated subunit influenza	Inactivated trivalent influenza vaccine	Influenza	Nasal	Nasal immunization with chitosan plus trivalent inactivated influenza is a potentially effective, easily-administered form of vaccination	Read et al. (2005)
Mono-*N*-carboxymethyl chitosan (MCC) and *N*-trimethyl chitosan (TMC)	Nanoparticles	Tetanus toxoid	*Clostridium tetani*	Nasal	Chitosan and TMC nanoparticles which have positively charged surfaces induced higher serum IgG titers when compared to those prepared with MCC which are negatively charged and smaller in size	Sayin et al. (2008)
N-trimethyl chitosan (TMC)	nanoparticles	Subunit antigen	influenza	Nasal	TMC nanoparticles are a potent new delivery system for i.n. administered influenza antigens	Amidi et al. (2007b)
Pluronic F127/ chitosan	Combination	Tetanus toxoid	*Clostridium tetani*	Nasal	F127/chitosan represents a novel mucosal vaccine delivery system, consisting of two components, that appear to exert an additive or synergistic effect on the immune	Westerink et al. (2001)

(*continued*)

TABLE 25.1 (continued)
List of Chitosan-Based Carrier Developed for Vaccine Delivery either Alone or in Combination with Other Materials

Chitosan/Chitosan with Other Material	Carrier	Antigen	Microbe/Disease	Route	Remark	References
Mannosylated chitosan	Microparticles	*Bordetella bronchisepti*ca antigens containing dermonecrotoxin (BBD)	*Bordetella bronchiseptica*	Nasal	Mannose moieties in the MCMs enhanced immune-stimulating activities through mucosal delivery due to a specific interaction between mannose groups in the MCMs and mannose receptors on the macrophages	Jiang et al. (2008)
Chtiosan	Microspheres	*Bordetella bronchisepti*ca antigens	Atrophic rhinitis	Nasal	Mucosal and systemic immune responses induced in the current study seemed to indicate that the intranasal administration of BBD-CMs may be an effective vaccine against atrophic rhinitis in pigs	Kang et al. (2007)
Chitosan-modified PLGA	Microspheres	Recombinant antigen	Hepatitis B	Nasal	Modified PLGA microspheres (cationic microspheres) thus produced humoral (both systemic and mucosal) and cellular immune responses upon nasal administration	Jaganathan and Vyas (2006)
Chitosan and trimethylchitosan	Microparticles or powder suspensions	LTK63 mutant	Meningococcal C conjugate	Nasal	Intranasal but not parenteral immunization, induced bactericidal antibodies at the nasal level, when formulated with both delivery system and adjuvant	Baudner et al. (2003, 2004, 2005)
Chitosan as mixture	Insufflations	*Neisseria meningitidis* serogroup C polysaccharide-CRM197 conjugate	Meningococcal bacteria and diphtheria	Nasal	Simple nasal insufflation of existing MCP-CRM197 conjugate vaccines in chitosan offers an inexpensive but effective needle-free prime and boost against serogroup C *N. meningitidis* and diphtheria	Huo et al. (2005)

Chitosan	Combination with muramyl di-peptide (MDP)	Recombinant urease	Helicobacter pylori	Nasal and intramuscular	Recognize the benefit of improved delivery of MDP intranasally due to the specific physiological effects of chitosan	Moschos et al. (2005)
Chitosan	Combination	Genetically detoxified diphtheria toxin (CRM197)	Diphtheria	Nasal	Vaccines capable of inducing strong Th2-type responses, such as CRM197 formulated with chitosan, have potential for the development of a protective mucosal vaccine against diphtheria in humans	McNeela et al. (2000, 2004)
Chitosan	Complex	Plasmid DNA	Respiratory syncytial virus (RSV)	Nasal	Following RSV challenge of chitosan/DNA immunized mice, a significant reduction ($P < 0.001$) in the virus load was observed in the lungs of immunized mice compared to that in the control group	Iqbal et al. (2003)
Chitosan	Particle and chitosan coated emulsion	ovalbumin (OVA) and cholera toxin (CT)	Immunization potential study	Nasal and intraperitoneal	CC-Emul and the smaller-size (0.4 microm) NP are effective for targeting to nasal-associated lymphoid tissues (NALTs) in nasal vaccine delivery	Nagamoto et al. (2004)
Chitosan	Nanoparticles	Plasmid DNA	Immunization potential study	topical	Chitosan-based nanoparticles containing pDNA resulted in both detectable and quantifiable levels of luciferase expression in mouse skin 24 h after topical application, and significant antigen-specific IgG titer to expressed beta-galactosidase at 28 days	Cui and Mumper (2001)
Chitosan	Nanoparticle	Plasmid DNA	Hepatitis B	Nasal	Significant potential of chitosan nanoparticles as DNA vaccine carrier and adjuvant for effective immunization through non-invasive nasal route was observed.	Khatri et al. (2008a)

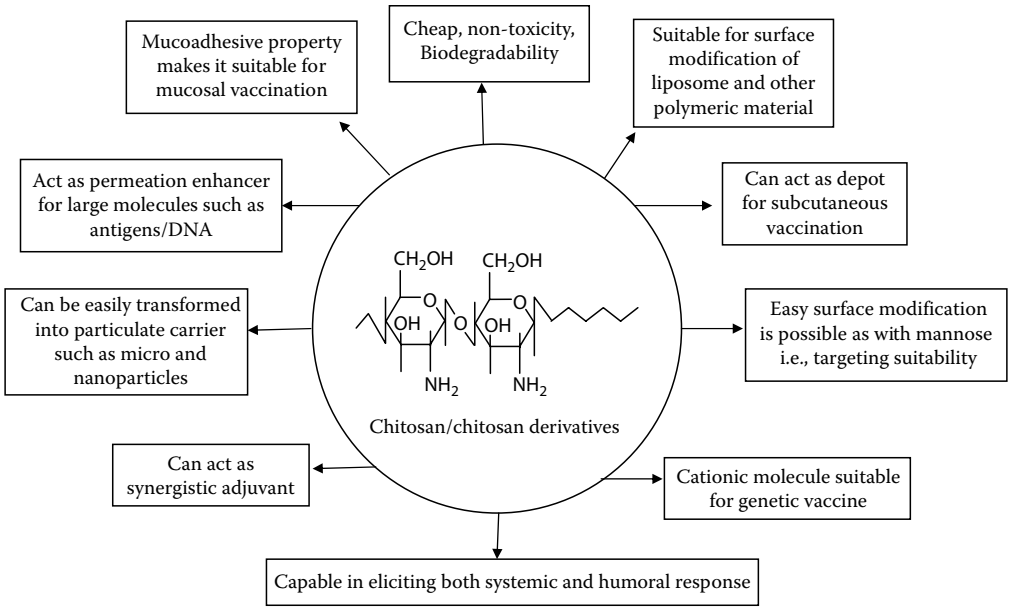

FIGURE 25.2 Properties of chitosan and chitosan derivatives which makes them suitable vehicle/carrier constructs for vaccine delivery.

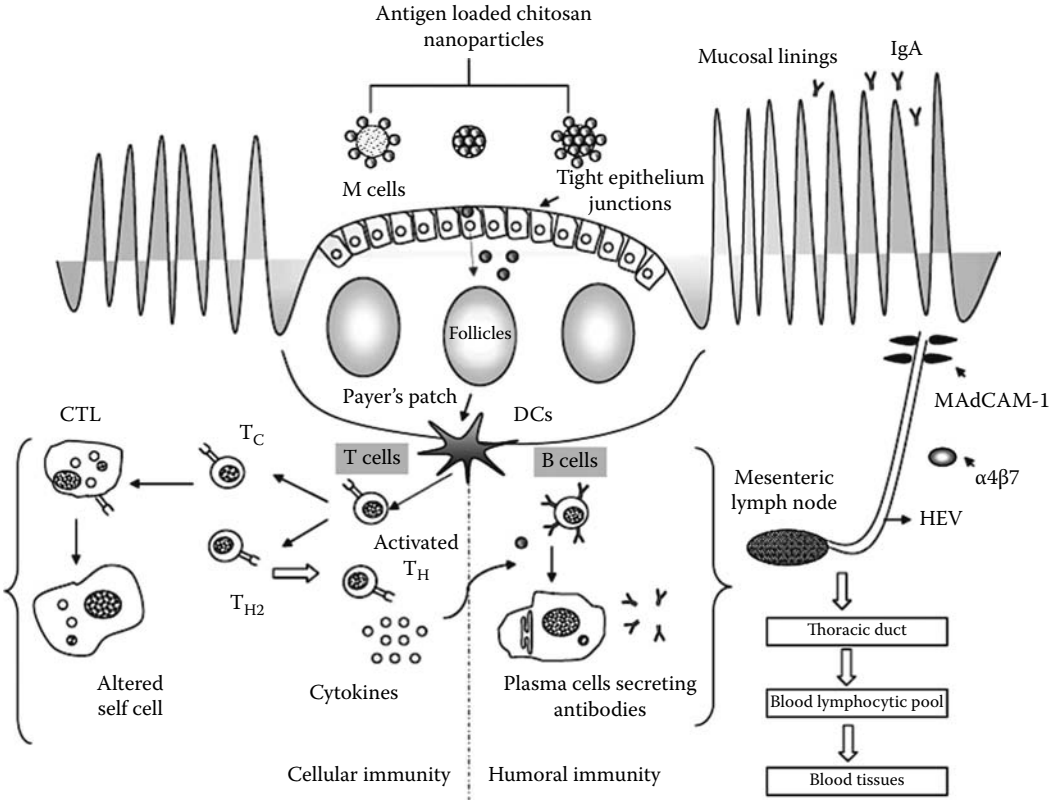

FIGURE 25.3 Schematic presentation of sequential immunological events occurred at mucosal surface particularly M cells after uptake and processing of antigen loaded chitosan nanoparticles and generation of immune response.

(Bramwell and Perrie 2006; Fu et al. 2006; Chen et al. 2008a,b). The common processes used to encapsulate antigen within chitosan matrixes include such as ionotropic gelation, spray drying, emulsification-solvent evaporation, and coacervation phase separation. Sometimes, combinations of these processes can also be used in order to obtain microparticles with specific size, shape, and properties. The chitosan-encapsulated tetanus toxoid (CS-TT) microparticles, for example, developed by ionic cross-linking using sodium tripolyphosphate (TPP) could elicit systemic and local immune response as estimated and reported (Ahire et al. 2007). Enhancement in the systemic and local immune responses was recorded as a compared response following the oral administration of TT in PBS. Chitosan microparticles for mucosal vaccination against diphtheria via oral and nasal routes were tested and significant systemic as well as humoral immune responses were recorded, demonstrating that chitosan microparticles are highly promising carrier mucosal vaccine delivery systems. Figure 25.3 shows the immunological consequences generated at mucosal lining after uptake and processing of chitosan nanoparticles loaded with antigenic material.

25.4 CHITOSAN AND OTHER CARRIERS FOR IMMUNIZATION: A RELATIVE ACCOUNT

The important issue in vaccine delivery is that the selected carrier must possess immunoadjuvanticity so that it can enhance the effect of antigen and as a result in eliciting immune response. Since there are numbers of materials available that possess adjuvanticity, the question is what relative advantageous applications do chitosan provide. PLGA is also one of the important materials used for vaccine delivery. Though the immune responses with PLGA are promising, it is expensive and therefore finds limited use. As an alternative option to PLGA is chitosan. The relative performances of microspheres of chitosan and PLGA loaded with tetanus toxoid were evaluated as single dose formulation in our laboratory (Jaganathan et al. 2005). The results indicated that a single injection of PLGA and chitosan microspheres containing TT could maintain the antibody response at a level comparable to the one obtained following booster injections of conventional alum-adsorbed vaccines. The PLGA and chitosan microspheres were fairly stable vaccine formulations capable of eliciting an equal immune response (Figure 25.4). Hence chitosan can be used in place of relatively expensive polymer i.e., PLGA. Figure 25.5 shows the morphological examination of TT-loaded chitosan microspheres.

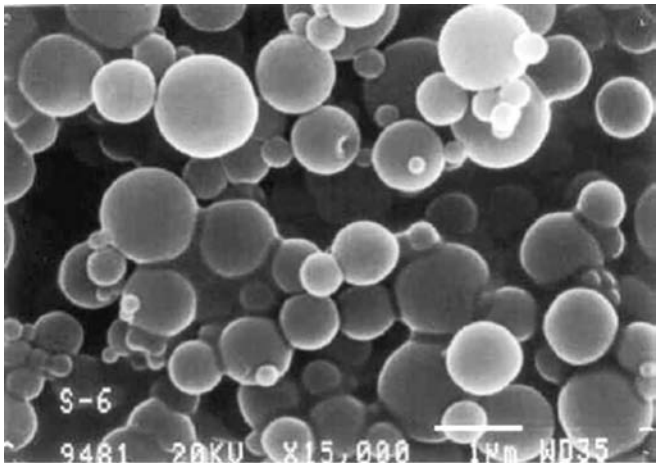

FIGURE 25.4 SEM photograph and stabilized TT-chitosan microspheres. (From Jaganathan, K.S. et al., *Int. J. Pharm.,* 294, 23, 2005. With permission.)

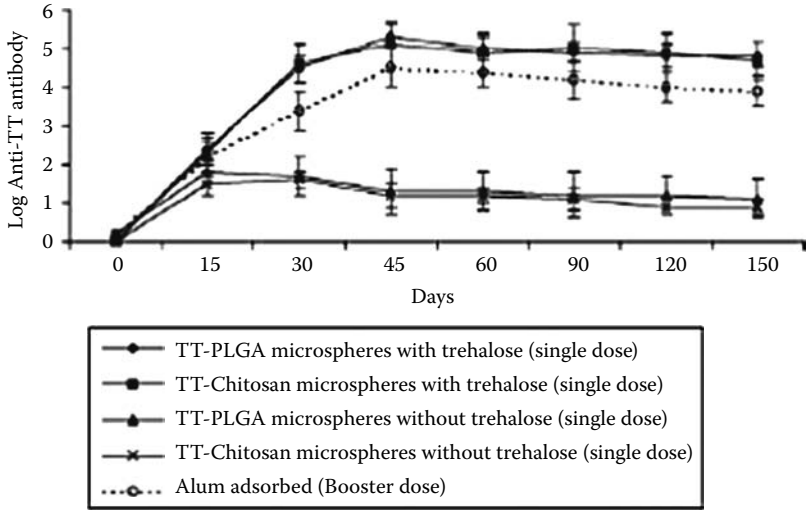

FIGURE 25.5 Comparitive Anti-TT antibodies response of TT-PLGA and TT-chitosan microspheres based formulations. (From Jaganathan, K.S. et al., *Int. J. Pharm.,* 294, 23, 2005. With permission.)

25.5 COMBINATION OF CHITOSAN WITH OTHER POLYMERS/LIPIDS FOR IMMUNIZATION

Chitosan has been used as mucoadhesive material, co-adjuvant, and penetration enhancer with liposome, multiple emulsions, and other polymeric particulate carriers. The adenoviral vectors (AdV) microencapsulated in chitosan microspheres has been achieved by ionotropic coacervation of chitosan using bile salts as counteranion (Lameiro et al. 2006). High encapsulation efficiency, i.e., more than 84% resulted in the formulations that were prepared using sodium deoxycholate as an ionotropic counteranion and Pluronic F68 as a dispersant. The formulations developed were able to maintain AdV infectivity and mitigate the release mediated through biodigestion (in situ) of the microparticles by the cell monolayers. The combination of bile salt and chitosan showed good properties for mucosal-attenuated adenovirus delivery. Similarly promising protection was recorded in the case of model protein antigen BSA when administered in chitosan–bile salt-based system. It was estimated that nearly 55% loaded antigen remained following an initial burst release of the content when studied in PBS pH 7.4 and 0.1 N HCl (Lameiro et al. 2006). Pandit et al. (2007) observed higher immune response of HBsAg-loaded PLA microsphere when combined with chitosan as compared to plain PLA microsphere.

25.6 RATIONALITY OF CHITOSAN IN IMMUNIZATION VIA VARIOUS ROUTES OF DELIVERY

25.6.1 Parenteral Immunization

Biodegradability, immunological activity, and high viscosity position chitosan as an excellent candidate for use as a depot/adjuvant for parenteral vaccination (Ghendon et al. 2008, 2009). Zaharoff et al. (2007a,b) reported that chitosan enhanced antigen-specific antibody titers over fivefold and antigen-specific splenic CD4+ proliferation over sixfold when given with model antigen (Zaharoff et al. 2007a,b). Significant increases in antibody titers together with remarkable delayed-type hypersensitivity (DTH) responses revealed that chitosan induce both humoral- and cell-mediated immune responses. When compared with traditional vaccine adjuvants, chitosan was found to be equipotent to incomplete Freund's adjuvant (IFA) and even superior to aluminum hydroxide. The studies suggested

that chitosan exhibited at least two characteristics that are responsible for its adjuvancity. Firstly, the viscous chitosan solution serves as an antigen reserve. More specifically, less than 9% of a protein antigen, when delivered in saline, remained at the injection site after 8 h. However, in case of chitosan-carried antigen more than 60% of the protein antigen remained at the injection site for 7 days. Secondly, chitosan induced a transient cellular expansion (nearly 67%) in draining lymph nodes. The expansion peaked between 14 and 21 days after chitosan injection and decreased as the polysaccharide degraded. These studies, taken together with the enhancement of immunological response, demonstrate that chitosan is a promising and safe platform for parenteral vaccine delivery. In another study, the ability of chitosan to (1) control the dissemination of a cytokine, GM-CSF and (2) enhance the immunoadjuvant properties of GM-CSF has also been evaluated by Zaharoff et al. (2007b). They reported that chitosan solution, due to its high viscosity, could retain coformulated rGM-CSF at the site of injection. The adjuvant ability of chitosan/rGM-CSF was compared with chitosan solution and rGMCSF alone. When delivered in a saline vehicle, rGM-CSF was undetectable up to 12–24 h. In contrast, chitosan solution could maintain a measurable depot of recombinant GM-CSF (rGM-CSF) at a subcutaneous injection site for up to 9 days. Furthermore, lymph node cells from mice injected with chitosan/rGM-CSF (20 μg) induced greater allogeneic T cell proliferation, indicating increased magnitude of antigen processing and presenting capability compared to lymph node cells from mice injected with rGM-CSF alone. Altogether, chitosan/rGM-CSF outperformed the standard rGM-CSF administrations with regard to dendritic cell recruitment, antigen presentation, and vaccine enhancement (Zaharoff et al. 2007b).

25.6.2 Oral Immunization

Oral immunization offers the safest and most convenient mode of vaccination and categorically induces mucosal immunity. However, it has not been successful to date because of the poor antigenic processing and presentation to processing sites and also due to antigen instability in the gastrointestinal tract (Jain et al. 2006). Orally administered antigens are mostly degraded in the acidic environment of stomach and by proteolytic enzyme–inactivating enzymes of the stomach before they could reach the M-cells of the Peyer's patches in the gastrointestinal tract (Borges et al. 2005). Although oral vaccination has numerous advantages over parenteral injection, degradation of the vaccine in the gut and low uptake in the lymphoid tissue of the gastrointestinal tract still complicate and confront the development of oral vaccines. The uptake of particulate antigen carrier systems by specialized M-cells of the gut-associated lymphoid tissue remains yet another limiting constituent in inducing efficient immune responses after oral vaccination (Borges et al. 2006). Table 25.2 provides list of some of the important literature related to M cell uptake and processing of particulate carrier.

Chitosan-based carrier system moreover showed a promising response. They are effectively taken up by M-cells and transported together with contained antigen to the presentation site. The ovalbumin-loaded chitosan microparticles are reportedly taken up by the Peyer's patches of the gut-associated lymphoid tissue (GALT). This unique uptake process demonstrates that chitosan particulate drug carrier systems are highly promising for oral vaccination in particular. In order to develop effective chitosan-based formulation, few approaches have been developed, which are discussed in the following sections and are shown schematically in Figure 25.6.

25.6.2.1 Sodium Alginate–Coated Chitosan Nanoparticles for Oral Immunization

For oral immunization, chitosan in the form of micro- and nanoparticles have been routinely explored; however, the solubility of chitosan at low acidic pH imposes problems when administered orally. In order to circumvent this hurdle, a number of strategies have been proposed and developed. Olga Borges et al. (2006) developed sodium alginate–coated chitosan nanoparticles loaded with model antigen ovalbumin. These particles were designed for delivery of antigen to the lymphoid tissue (MALT) in particular to or through the ileal Peyer's patches and also to assess the in vivo release profiles of the antigen from coated nanoparticles in order to examine possible interactions

TABLE 25.2
List of Important Literature Related to M Cell Uptake of Particulate Carrier

Title	Article Type	Reference
Particle uptake by Peyer's patches: a pathway for drug and vaccine delivery.	Review	Shakweh et al. (2004)
Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting.	Review	Brayden et al. (2005)
Targeting of mucosal vaccines to Peyer's patch M cells.	Review	Frey and Neutra (1997)
M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis.	Review	Corr et al. (2008)
Peyer's patches: organized lymphoid structures for the induction of mucosal immune responses in the intestine.	Review	Makala et al. (2002–2003)
In vivo uptake of chitosan microparticles by murine Peyer's patches: visualization studies using confocal laser scanning microscopy and immunohistochemistry.	Research	van der Lubben et al. (2001a)
Transport of chitosan microparticles for mucosal vaccine delivery in a human intestinal M-cell model.	Research	van der Lubben et al. (2002)
Microparticle transport in the human intestinal M cell model.	Research	Lai and D'Souza (2008)
M cell-targeted delivery of vaccines and therapeutics.	Review	Kuolee and Chen (2008)
Apical membrane receptors on intestinal M cells: potential targets for vaccine delivery.	Review	Brayden and Baird (2004)
Chitosan microparticles for oral vaccination: preparation, characterization and preliminary *in vivo* uptake studies in murine Peyer's patches.	Research	van der Lubben et al. (2001c)

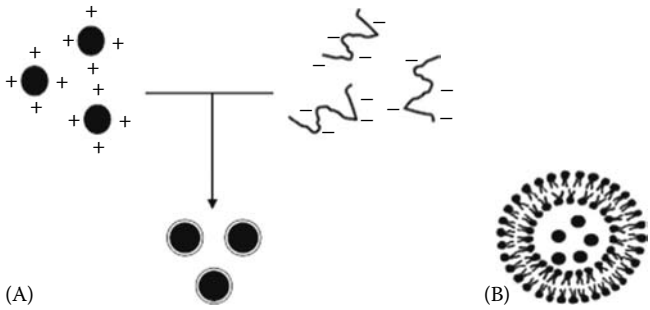

FIGURE 25.6 Different approaches which have been developed for protection of chitosan-based carriers loaded with antigen from harsh environment of stomach (A) protected alginate coated chitosan nanoparticles; (B) encapsulation of chitosan nanoparticles within vesicular systems.

between the chitosan core and the alginate coating that affects release. The cytotoxicity of the coated and uncoated nanoparticles in comparison to the plain polymers was also evaluated. The adsorption of therapeutic proteins and model antigens onto chitosan nanoparticles was found to be a very mild (which does not affect antigen instability) process with excessively high loading efficacy. Sodium alginate–coated particles nanometric in size with a much better stability and controlled release properties were tested to be efficient for vaccine delivery as compared to chitosan-loaded cores. It was also documented that the preparation process of the nanoparticles had been safe, easy, and reproducible. Nevertheless, a low grade stimulation of the splenocytes cocultured with the nanoparticles was recorded. These hydrophilic-coated nanoparticles even with a negative surface charge are taken up by rat Peyer's patches, which made them promising carriers for mucosal vaccination. A similar study was carried out using chitosan microparticles coated with alginate by

Li et al. (2008). The prepared alginate-coated chitosan microparticles, with mean diameter of about 1 μm, were evaluated for mucosal vaccine. Moreover, alginate coating as carrier could modulate the release behavior of BSA from alginate-coated chitosan microparticles and could effectively protect model protein (BSA) from degradation in acidic medium in vitro for at least 2 h.

25.6.2.2 Chitosan Nanoparticles in Vesicular Systems for Oral Immunization

The encapsulation of chitosan nanoparticles within liposomes and niosomes for its protection from low pH gastric medium was achieved by our group (Jain et al. 2006). It was hypothesized that though phospholipids or surfactants in vesicular form are thought to be unstable in the gastrointestinal environment, it has been reported that gastric lipases do not hydrolyze phospholipids or lipoidal surfactants. The digestion of these lipids takes place mainly in the small intestine by pancreatic lipase, colipase, phospholipase A2 and cholesterol esterase and by the action of bile salts. Thus, vesicular systems apparently provide protection to chitosan nanoparticles in the stomach, while in the intestine, the particles may get or remain encapsulated sequestered and taken by M-cells of the Peyer's patches due to their size as well as bioadhesive nature.

Antigen contained in chitosan within vesicular systems could elicit both systemic and mucosal immune responses remarkably and hence could be a promising carrier system for a number of antigens other than BSA (Jain et al. 2006). With the identification of such types of carrier systems, which have the potential to elicit systemic immunity following oral administration, a new means of development of cost-effective formulations of potential vaccines may emerge and find wide application.

25.6.3 Nasal Immunization

The nasal mucosa is an attractive site for the delivery of vaccines and has certain advantages over other sites. It is highly vascularized, possesses a relatively large absorptive surface, and displays a low proteolytic activity. Importantly, nasal immunization can induce both mucosal and systemic immune responses (Jabbal-Gill et al. 1998; Ahire et al. 2007). The major factors limiting the absorption of nasally administered vaccines are the poor ability of antigen/protein to cross nasal barriers and the mucociliary clearance of carrier from the nasomucosal site, thus the soluble antigens are removed from the nasal cavity. Chitosan particles are mainly taken up by M cells of the NALT, which are capable of sampling and transporting antigen across the cell through transcytosis without degradation (Khatri et al. 2008a,b). Hence, chitosan and TMC may be employed for nasal vaccine delivery in both solution and particulate forms (Jabbal-Gill et al. 1998; Garmise et al. 2007). Chitosan, being a soluble biopolymer, can be successfully used for enhancing nasal delivery of various antigens as well as DNA vaccine (Khatri et al. 2008a,b). The study related to the uptake of microparticle of fluorescent material through M cells of nasopharyngeal lymphoid tissue (NALT), considered to be playing an important role for vaccine delivery systems in humans, revealed that NALT-M cells possesses high uptake ability as smallest particles, 0.2 μm in diameter, compared with those with a diameter of 0.5, 1.0, or 2.0 μm. It was also appreciated that surface coatings with chitosan resulted in yet higher uptake into the NALT (Khatri et al. 2008a,b). These results are indicative of the fact that nasal administration of antigenic microparticles, which were coated with chitosan, probably leads to a useful method of transnasal vaccination against respiratory and intestinal infections in humans.

We have investigated the potential of chitosan nanoparticles also as a DNA vaccine carrier and adjuvant for effective immunization through noninvasive nasal route against hepatitis B (Khatri et al. 2008a). The nasal administration of chitosan nanoparticles resulted in serum anti-HBsAg titer that was less compared to that elicited by naked DNA and alum-adsorbed HBsAg; however, the mice become seroprotective within 2 weeks and the immunoglobulin level was above the clinically protective level. However, intramuscular administration of naked DNA and alum-adsorbed HBsAg did not elicit sIgA titer in mucosal secretions that was induced by nasal immunization with chitosan nanoparticles. Similarly, cellular responses (cytokine levels) were poor in case of alum-adsorbed

HBsAg. Chitosan nanoparticles thus produced humoral (both systemic and mucosal) and cellular immune responses upon nasal administration.

In another study, we investigated the potential utility of glycol chitosan coated liposomes as a nasal vaccine delivery vehicle for eliciting viral-specific humoral, mucosal and cellular immune responses (Khatri et al. 2008b). Following intranasal administration, glycol chitosan–coated liposomes elicited humoral mucosal and cellular immune responses that were significant as compared to naked DNA, justifying the potential advantage of mucosal vaccination in the production of local antibodies IgA at the sites where pathogens enter the body. Say1n et al. (2008) developed the nanoparticulate systems using chitosan derivatives using different charge density, *N*-trimethyl chitosan (TMC, polycationic), and mono-*N* carboxymethyl chitosan (MCC, polyampholytic) as an antigen carrier vehicle for intranasal immunization. Significantly enhanced immune responses were obtained with intranasal (i.n.) application of nanoparticle formulations. Chitosan and TMC nanoparticles with positively charged surfaces could induce higher serum IgG titers compared to those based on MCC, which are negatively charged and smaller in size.

It has been reported that molecular weights (MW) of chitosan exhibited an influence on the level or magnitude of immune response against model antigen when administered with chitosan through the nasal route. Boonyo et al. (2008) studied chitosan(s) of different molecular weights and trimethyl chitosan derivatives as particles for nasal immunization using OVA as model antigen. The molecular weight of the chitosan in the studied range did not present a significant influence on the characteristics of the particles. All TMC particles showed higher zeta potential, smaller size, higher loading efficiency, and faster release profile than chitosan particles.

High-molecular-weight (M_w) chitosan (CS) solutions have already been proposed as vehicles for nasal immunization. The potential utility of low M_w CS in the form of nanoparticles as new long-term nasal vaccine delivery vehicles has been investigated by Vila et al. (2004) using tetanus toxoid as a model antigen. They found that the ability of chitosan nanoparticles to provide improved access to the associated NALT was not significantly influenced by the CS M_w. Indeed, high and long-lasting responses could be obtained using low-M_w CS molecules.

25.7 TARGETING POTENTIAL OF CHITOSAN-BASED CARRIERS IN IMMUNIZATION

Chitosan has been explored for targeted delivery of antigens/pDNAs to antigen-presenting cells (APCs) as a coating material. In our laboratory, we have developed the glycol chitosan-coated liposomes containing plasmid DNA encoding for surface protein of Hepatitis B virus (Khatri et al. 2008b). To investigate the interaction of glycol chitosan–coated liposomes with the nasal tissue, the liposomes were labeled with FITC-BSA. The fluorescent microscopy studies revealed that both glycol chitosan–coated liposomes and uncoated liposomes adhered to the mucosal tissue and may subsequently be taken up by the nasal epithelium cells. It is reasonable to suggest that they could have further transported to the submucosa layer. However, the extent of adherence was more in case of glycol chitosan–modified liposomes as shown in Figure 25.7. Also, the immunization potential on nasal administration of glycol chitosan–modified liposomes resulted in serum anti-HBsAg titer that was low compared to that elicited by naked DNA and alum-adsorbed HBsAg administered intramuscularly, but the mice were seroprotective within 2 weeks and the antibody titer was significantly higher compared to the level estimated after naked DNA was administered intranasally.

Fischer et al. (2006) developed a novel surfactant-free, one-step process for the concomitant formation of PLGA microparticles and their surface coating with chitosan, which can be further utilized for the attachment of the ligands (as in this case they used fluorescamine and NHS-PEG-biotin) for delivery of antigens (Fischer et al. 2006). They suggested that targeted delivery of antigen is possible with chitosan-modified PLGA particles using appropriate ligands for targeting the various cells of immune system. Chitosan anchored with mannose moieties for macrophage targeting has

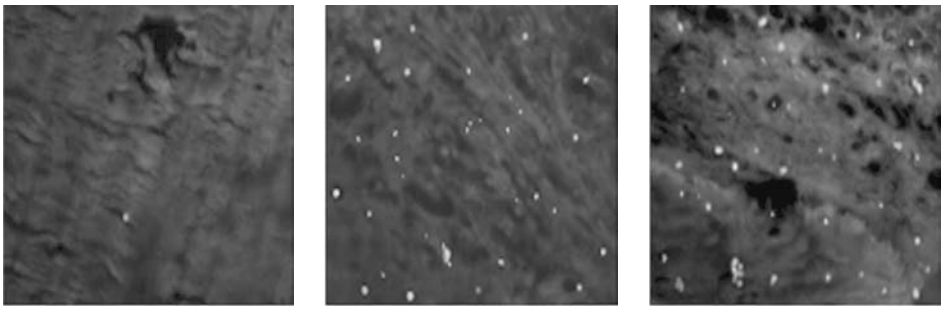

FIGURE 25.7 Fluorescence micrographs of the nasal tissue of rats excised following administration of (A) FITC-BSA solution; (B) FITC-BSA encapsulated in plain liposomes or (C) FITC-BSA encapsulated in glycol chitosan coated liposomes. (From Khatri, K. et al., *Vaccine*, 26, 2225, 2008. With permission.)

been tested. Recently, *Bordetella bronchiseptica* antigens containing dermonecrotoxin (BABD)-encapsulated mannosylated chitosan microspheres (MCMs) have been developed by Jiang et al. (2008). Fluorescence confocal microscopy indicated that BBD-loaded MCMs (BBD-MCMs) bound to mannose receptors in murine macrophages (RAW264.7 cells). In addition, mice intranasally immunized with BBD-MCMs showed significantly higher BBD-specific IgA antibody responses in saliva and serum compared with mice immunized with BBD-loaded plain chitosan microspheres.

25.8 CONCLUSION

Chitosan and its derivatives, either as particulate carrier or as mixture with other adjuvants, could serve as an excellent adjuvant for the delivery of antigen as well as plasmid DNA and capable of generating high immunological response. The degree of deacetylation and molecular weight, however, affect the responses and immunogenic capacity of chitosan. The safe biodegradation of chitosan is responsible for its great potential use among other biomaterials. A precise performance being amenable to surface modification particularly when chitosans are fabricated as a particulate carrier in the form of nanoparticles or microparticles holds great promise for future application.

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26 Chitosan-Conjugated DNA Nanoparticle Delivery Systems for Gene Therapy

R. Jayakumar, K. P. Chennazhi, S. V. Nair, Tetsuya Furuike, and Hiroshi Tamura

CONTENTS

26.1 INTRODUCTION

Gene therapy is the treatment of human disorders by the introduction of genetic material into specific target cells of a patient, in which production of the encoded protein occurs (Corsi et al. 2003). A wide variety of vectors that are capable of delivering therapeutic genes into the desired target cells have been studied (Nishikawa and Hashida 2002). Gene therapy is currently being applied to many different health problems such as cancer, AIDS, and cardiovascular diseases (Ozbas-Turan et al. 2003). Several trials employing gene therapy protocols have already been successfully completed in patients with cystic fibrosis (Gill et al. 1997, Porteous et al. 1997) and adenosine deaminase deficiency (Bordignon et al. 1995). Many researches are working in the field of gene delivery to develop ideal gene delivery carriers. There are several systems that can be used to transfer foreign genetic material into the human body. Gene carriers should possess certain ideal properties. The DNA to be transferred must escape the processes that affect the disposition of macromolecules. These processes include the interaction with blood components, vascular endothelial cells, and uptake by the reticuloendothelial system. Furthermore, the degradation of therapeutic DNA by serum nucleases is also a potential obstacle for functional delivery to the target cell (Quong and Neufeld 1998). Thus, an ideal gene-delivering carrier should transport genetic materials without any toxicity and immune responses (Smith et al. 1997). It must be capable of protecting the DNA until it reaches its target. To do so, the system must be small enough to allow internalization into cells and passage to the nucleus, it must have flexible tropisms for applicability in a range of disease targets, and it must be capable of escaping endosome–lysosome processing and of following endocytosis (Mansouri et al. 2004).

Currently, transport of exogenous DNA to cells can be achieved using viral and nonviral vectors or as naked DNA. The simplest nonviral gene delivery system simply uses naked DNA. The overall level of expression is much lower with naked DNA than with either viral or liposomal vectors. Naked DNA is also unsuitable for systemic administration due to the presence of serum nucleases.

As a result, direct injection of DNA seems to be limited to tissues that are easily accessible by direct injection such as the skin and the muscle (Mansouri et al. 2004). The most common viral vectors used today are retrovirus, herpes simplex virus, lentivirus, adenovirus, and adeno-associated virus, each having their own characteristics (Oligino et al. 2000). Although viral vectors have obvious advantages such as high transfection rate and a rapid transcription of the foreign material inserted in the viral genome, there are some evident drawbacks limiting their clinical use, such as poor target specificity, low capacity to incorporate foreign DNA sequences to their genome (Mansouri et al. 2004), toxic and inflammatory effects, eliciting immune responses, viral wild-type mutations, and potential oncogenic effects (Lee et al. 1998). However, the use of viruses in gene therapy could be limited by various factors. First and foremost, security issues have been raised following the death of a patient during a clinical trial that investigated the potential of gene therapy using viral vectors (Somia and Verma 2000, Ferber 2001). Second, gene therapy using viral vectors is limited by the fact that only small sequences of DNA can be inserted in the virus genome. Third, viruses present a variety of potential problems to the patients such as toxicity, immune responses, and inflammatory responses. Lastly, insertional mutagenesis and oncogenic effects can occur when used in vivo (Lee et al. 1998).

Nonviral vectors have the potential to be administered repeatedly with minimal host immune response, are targetable, stable in storage, and easy to produce in large quantities (Mao et al. 2001). The nonviral vectors include cationic molecules such as cationic lipids and synthetic or natural cationic polymers, which are widely used to condense DNA and to efficiently deliver therapeutic genes within mammalian cells (Rolland 1998, Garnett 1999). Because of their permanent cationic charge, these interact electrostatically with negatively charged DNA and form complexes (lipo- or polyplexes). Although cationic lipids have low immunogenicity (Deshpande et al. 1998), they are not considered for gene therapy due to their toxicity and the relatively low transfection efficiency. DNA–polymer complexes involving cationic polymers, on the other hand, are more stable than cationic lipids (De Smedt et al. 2000), though the efficiency of gene delivery by cationic polymers is still relatively low (Rolland 1998). Several cationic polymers have been investigated that lead to increased transfection efficiencies (Somia and Verma 2000, Ferber 2001). They show structural variability and versatility including the possibility of covalent binding of targeting moieties for gene expression mediated through specific receptors (Somia and Verma 2000, Liu and Yao 2002). Cationic polymers are able to condense more DNA than lipids. They form complexes with DNA and protect it against nuclease degradation (Gao and Huang 1996). The cationic polymers that has been investigated include poly(D,L-lactide-*co*-glycolide) (PLGA) (Tahara et al. 2008), poly(ethylene imine) [PEI], poly(L-lysine), polybrene, tetraminofullerene, poly(L-histidine)-graft-poly(L-lysine) (Mansouri et al. 2004), etc. Natural polymers that have been tried include chitosan and its derivatives, collagen, gelatin etc. (Dang and Leong 2006).

Polymeric nanospheres have been used to deliver medicines because of their advantages such as high stability, easy uptake into the cells by endocytosis, and targeting ability to specific tissues or organs by adsorption or binding with ligand at the surface of the particles (Lobenberg et al. 1997). In particular, biodegradable nanospheres are available for delivering drugs and degraded after passing required specific site (Belbella et al. 1996). Among them poly(lactide) (PLA) and poly(D,L-lactide-*co*-glycolide) (PLGA) have been approved by the Food and Drug Administration (FDA) for certain human clinical uses (Sahoo et al. 2002). There are two types of nanoparticle system carrying nucleic acid, i.e., DNA or RNA entrapping system (Wang et al. 1999) and surface binding system (Kim et al. 2005). Surface binding systems utilize an ionic interaction between cationic polymer and the anionic nucleic acid. Nucleic acid entrapping system is a reservoir-type nanosphere system. This system has the advantages of nucleic acid protection. The application of DNA–chitosan nanospheres has advanced in vitro DNA transfection research and data have been accumulating that shows their usefulness for gene delivery (Erbacher et al. 1998, Corsi et al. 2003). Of these, chitosan and its derivatives have received much attention because of their excellent biocompatibility, low immunogenicity, and reduced

cytotoxicity compared to other polymers (Erbacher et al. 1998, Roy et al. 1999, Mao et al. 2001, Liu and Yao 2002). Chitosan, a cationic polysaccharide obtained by alkaline *N*-deacetylation of chitin, is one of the most widely utilized polysaccharides (Romoren et al. 2002). It is a nontoxic biodegradable polymer with low immunogenicity (Muzzarelli 1973). These characteristics make chitosan an excellent candidate for various biomedical applications such as drug delivery, tissue engineering, and gene delivery (Illum 1998, Roy et al. 1999, Hoggard et al. 2001, Ishii et al. 2001, Lee et al. 2001, Thanou et al. 2001, Ozbas-Turan et al. 2002, Khor and Lim 2003, Kumar et al. 2003, Chen et al. 2004, Kai and Ochiya 2004). It is a good candidate for gene delivery system because positively charged chitosan can be complexed with negatively charged DNA (Mac Laughlin et al. 1998, Richardson et al. 1999, Fang et al. 2001). Chitosan and its derivatives have been complexed with DNA by ionic interactions between anionic phosphate backbones of DNA and primary amine groups of chitosan. This binding protects the DNA from nuclease degradation (Cui and Mumper 2001, Illum et al. 2001). Also, the mucoadhesive property of chitosan potentially permits a sustained interaction between the macromolecule being "delivered" and the membrane epithelia, promoting more efficient uptake (Takeuchi et al. 1996, Richardson et al. 1999, Hejazi and Amiji 2003), and chitosan has the ability to open intercellular tight junctions, facilitating its transport into the cells (Illum et al. 2001). It has advantages of not requiring sonication and organic solvents for its preparation, therefore minimizing possible damage to DNA during complexation. Various gene delivery systems based on chitosan has been described such as self-assembling polymeric and permanent oligomeric chitosan–DNA complexes, DNA–chitosan nanospheres, etc. (Borchard 2001).

In this review, we evaluated the preparation and application of chitosan-conjugated DNA and siRNA nanoparticles for gene delivery applications in detail.

26.2 CHITOSAN–DNA COMPLEX NANOPARTICLES

Polysaccharides and other cationic polymers have recently been used in pharmaceutical research and industry for their properties to control the release of antibiotics, DNA, proteins, peptide drugs, or vaccines, and they have also been extensively studied as nonviral DNA carriers for gene delivery and therapy. Among them, chitosan is the most used since it can promote long-term release of incorporated drugs. The preparation of chitosan and chitosan–DNA nanospheres by using a novel and simple osmosis-based method is recently been patented (Masotti et al. 2008). With this method, they were able to prepare chitosan–DNA particles of spherical morphology with an average diameter 38 ± 4 nm. Also, the DNA incorporation was pretty high (up to 30%) and the release process is gradual and prolonged in time. Another advantage of this method is that it can be used to easily modify the process by varying the solvent/nonsolvent couple, temperature, and membrane cut-off, affording useful nanostructured systems of different sizes and shapes that can be employed in several biomedical and biotechnological applications.

While looking back at the history of chitosan as a gene delivery system, there are many reports suggesting the advantages of chitosan as an efficient gene carrier molecule. The first report suggesting chitosan as the probable candidate for a gene delivery agent appeared in 1998 (Lee et al. 1998). The authors prepared a hydrophobically modified chitosan containing 5.1 deoxycholic acid groups per 100 anhydroglucose units by an EDC-mediated coupling reaction. The formation and characteristics of self-aggregates of hydrophobically modified chitosan were studied by fluorescence spectroscopy and dynamic light scattering method. Charge complex formation between self-aggregates and plasmid DNA was confirmed by electrophoresis on an agarose gel. The migration of DNA on an agarose gel was completely retarded above a charge ratio of 4/1 at pH 7.2. The free DNA dissociated from the complexes was observed by electrophoresis above pH 8.0 at a fixed charge ratio of 4/1. Chitosan self-aggregate–DNA complexes achieved an efficient transfection of COS-1 cells and the level of expression with plasmid–chitosan was observed to be no less than that with plasmid–lipofectin complexes in SOJ cells.

A few years later, Mao et al. reported preparation of chitosan–DNA nanoparticles using a complex cooperation process (Mao et al. 2001). They investigated important parameters for the nanoparticle synthesis, including the concentrations of DNA, chitosan and sodium sulfate, temperature of the solutions, pH of the buffer, and molecular weights of chitosan and DNA. At an amino group to phosphate group ratio (N/P ratio) between 3 and 8 and a chitosan concentration of 100 mg/mL, the size of particles was optimized to 100–250 nm with a narrow distribution, with a composition of 35.6% and 64.4% by weight for DNA and chitosan, respectively. The surface charge of these particles was slightly positive with a zeta potential of 112–118 mV at pH lower than 6.0, and became nearly neutral at pH 7.2. With this system, they found that the transfection efficiency of chitosan–DNA nanoparticles was cell-type-dependent. They also developed three different schemes to conjugate transferrin or KNOB protein to the nanoparticle surface. The transferrin conjugation only yielded a maximum of fourfold increase in their transfection efficiency in HEK293 cells and HeLa cells, whereas KNOB-conjugated nanoparticles could improve gene expression level in HeLa cells 130-fold. Conjugation of PEG on the nanoparticles allowed lyophilization without aggregation and without loss of bioactivity for at least 1 month in storage. The clearance of the PEGylated nanoparticles in mice following intravenous administration was slower than unmodified nanoparticles at 15 min, and with higher depositions in kidney and liver. However, no difference was observed at the 1 h time point.

In an approach to study the transfection mechanism of plasmid–chitosan complexes as well as the relationship between transfection activity and cell uptake, Ishii et al. (2001) used fluorescein isothiocyanate-labeled plasmid and Texas Red-labeled chitosan. They observed that several factors contribute to transfection activity: the molecular mass of chitosan, stoichiometry of complex, serum concentration, and pH of transfection medium. The level of transfection with plasmid–chitosan complexes was found to be highest when the molecular mass of chitosan was 40 or 84 kDa, ratio of chitosan nitrogen to DNA phosphate (N/P ratio) was 5, and transfection medium contained 10% serum at pH 7.0. While investigating the transfection mechanism, they found that plasmid–chitosan complexes most likely condense to form large aggregates, which absorb to the cell surface. After this, plasmid–chitosan complexes are endocytosed, and possibly released from endosomes due to swelling of lysosomes along with the swelling of plasmid–chitosan complex, causing the endosome to rupture. Finally, these complexes were observed to be accumulating in the nucleus.

Another approach of chitosan–DNA nanoparticles synthesis is by the complexation of the cationic polymer with a plasmid DNA (Corsi et al. 2003). The authors evaluated the transfection potential using three cell lines, viz. human mesenchymal stem cells (MSCs), human osteosarcoma cells (MG63), and human embryonic kidney cells (HEK293). DNA distribution within the nanoparticle was visualized by transmission electron and atomic force microscopy. The LipofectAMINETM 2000 (LF) reagent was used in comparison. The transfection of HEK293 cells is superior to that seen with MG63 cells and MSCs, however not surpassing that seen with LF. But most importantly, they found that the cytotoxicity was minimal with the chitosan–DNA nanoparticles compared to greater than 50% toxicity with LF. Their results suggest that chitosan–DNA nanoparticles have favorable characteristics for nonviral gene delivery, are cell-type-dependent and not cytotoxic.

Quaternized modifications of chitosan are another technique with characteristics that might be useful in DNA condensing and efficient gene delivery (Thanou et al. 2002). Quaternized chitosan oligomers were synthesized and the resulting complexes were characterized by photon correlation spectroscopy. Further, their ability to transfect COS-1 and Caco-2 cell lines in the presence and absence of fetal calf serum was investigated. The transfection efficiency was compared with DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium sulfate) lipoplexes. Additionally, their effect on the viability of the respective cell cultures was investigated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Their observations suggest that quaternized chitosan oligomers were able to condense DNA and form complexes with a size ranging from 200 to 500 nm. Chitoplexes proved to transfect COS-1 cells, however, to a lesser extent than DOTAP–DNA lipoplexes. The quaternized oligomer derivatives appeared to be superior to oligomeric chitosan.

The presence of fetal calf serum (FCS) did not affect the transfection efficiency of the chitoplexes, whereas the transfection efficiency of DOTAP–DNA complexes was decreased. Cells remained 100% viable in the presence of chitosan oligomers whereas viability of DOTAP treated cells decreased to 50% in both cell lines. Both DOTAP–DNA lipoplexes and chitoplexes resulted in less transfection efficiency in Caco-2 cell cultures than in COS-1 cells; however, quaternized chitosan oligomers proved to be superior to DOTAP. The effects on the viability of Caco-2 cells were similar to the effects observed in COS-1 cells. This report is also in line with the earlier one suggesting that chitosan–DNA complexes present suitable characteristics and less cytotoxic compared to lipid gene carriers and thus has the potential to be used as gene delivery vectors.

A self-assembled nanoparticle using a hydrophobically modified glycol chitosan for gene delivery has been prepared (Yoo et al. 2005). Here a primary amine of glycol chitosan was modified with 5β-cholanic acid to prepare a hydrophobically modified glycol chitosan (HGC). The modified chitosan was found to form DNA nanoparticles spontaneously by a hydrophobic interaction between HGC and hydrophobized DNA. As the HGC content increased, the encapsulation efficiencies of DNA increased while the size of HGC nanoparticles decreased. Upon increasing HGC contents, HGC nanoparticle became less cytotoxic. The increased HGC contents also facilitated endocytic uptakes of HGC nanoparticles by COS-1 cells. The HGC nanoparticles showed increasing in vitro transfection efficiencies in the presence serum. In vivo results also showed that the HGC nanoparticles had superior transfection efficiencies to naked DNA and a commercialized transfection agent. They concluded that the HGC nanoparticles composed of hydrophobized DNA and hydrophobically modified glycol chitosan played a significant role in enhancing transfection efficiencies in vitro as well as in vivo.

Another group used reverse microemulsion technique as a template to fabricate chitosan–alginate coreshell nanoparticles encapsulated with enhanced green fluorescent protein (EGFP)-encoded plasmids (You et al. 2006). These alginate-coated chitosan nanoparticles endocytozed by NIH 3T3 cells were found to trigger swelling of transport vesicles, which facilitate gene escape before entering digestive endolysosomal compartment, and concomitantly promote gene transfection rate. Their results indicate that DNA-encapsulated chitosan–alginate nanoparticles with average size of 64 nm (N/P ratio of 5) could achieve the level of gene expression comparable with the one obtained by using polyethyleneimine–DNA complexes.

As a novel technique, Hui et al. studied the gene delivery by chitosan–DNA nanoparticles through retrograde intrabiliary infusion (RII) and examined the efficacy of liver-specific targeting (Dai et al. 2006). The transfection efficiency of chitosan–DNA nanoparticles, as compared with PEI–DNA nanoparticles, was evaluated in Wistar rats by infusion into the common bile duct, portal vein, or tail vein. Chitosan–DNA nanoparticles administrated through the portal vein or tail vein did not produce detectable luciferase expression. In contrast, rats that received chitosan–DNA nanoparticles showed more than 500 times higher luciferase expression in the liver 3 days after RII and transgene expression levels decreased gradually over 14 days. Luciferase expression in the kidney, lung, spleen, and heart was negligible compared with that in the liver. RII of chitosan–DNA nanoparticles did not yield significant toxicity and damage to the liver and biliary tree as evidenced by liver function analysis and histopathological examination. Luciferase expression by RII of PEI–DNA nanoparticles was 17-fold lower than that of chitosan–DNA nanoparticles on day 3, but it increased slightly over time. These results suggest that gene delivery by chitosan–DNA nanoparticles through RII is a promising routine to achieve liver-targeted gene delivery and both gene carrier characteristics and mode of administration significantly influence gene delivery efficiency.

In another study designed to investigate the in vitro and in vivo transfection efficiency of chitosan nanoparticles as vectors for gene therapy, three types of chitosan nanoparticles [quaternized chitosan–60% trimethylated chitosan oligomer (TMCO-60%), C (43–45 kDa, 87%), and C (230 kDa, 90%)] were used to encapsulate plasmid DNA (pDNA) encoding green fluorescent protein (GFP) using the complex coacervation technique (Zheng et al. 2007). The in vitro study showed the transfection efficiency to be in the following descending order: TMCO-60% NC (43–45 kDa, 87%) NC

(230 kDa, 90%). TMCO-60% proved to be the most efficient and the optimal chitosan/pDNA ratio being 3.2:1. Further, they examined the in vivo transfection efficiency by feeding the chitosan–pDNA nanoparticles to 12 BALB/C-nu/nu nude mice and observed that the gastric and upper intestinal mucosa showed most prominent GPF expression. GFP expression in the mucosa of the stomach and duodenum, jejunum, ileum, and large intestine were found, respectively, in 100%, 88.9%, 77.8%, and 66.7% of the nude mice examined. TMCO-60%/pDNA nanoparticles exhibited better in vitro and in vivo transfection activity than the other two with minimal toxicity, which makes it a desirable nonviral vector for gene therapy via oral administration.

Weecharangsan et al. (2008), explored the differences in transfection levels of chitosan–DNA complexes formulated with various chitosan salts (CS) including chitosan hydrochloride (CHy), chitosan lactate (CLa), chitosan acetate (CAc), chitosan aspartate (CAs), and chitosan glutamate (CGl). CHy, CLa, CAc, CAs, and CGl, MW 45 kDa were found to form a complexes with pcDNA3-CMV-Luc at various N/P ratios. CGl/DNA complexes were formulated with various chitosan molecular weights (20, 45, 200, and 460 kDa). Gel electrophoresis illustrated that complete complexes formed at N/P ratios above 2 in all CS of MW 45 kDa. The transfection efficiency of CS–DNA complexes was found to be dependent on the salt form and molecular weight of chitosan, and the N/P ratio of CS/DNA complexes. For different CS complexes, the maximum transfection efficiency varied with different N/P ratios. CHy/DNA, CLa/DNA, CAc/DNA, CAs/DNA, and CGl/DNA complexes showed maximum transfection efficiencies at N/P ratios of 12, 12, 8, 6, and 6, respectively. Cytotoxicity results showed that all CS/DNA complexes had low cytotoxicity. This study suggests CS has the potential to be used as a safe gene delivery vector.

Chitosan, trimethyl chitosan, or polyethylenglycol-*graft*-trimethyl chitosan–DNA complexes were characterized for physicochemical properties such as hydrodynamic diameter, condensation efficiency, and DNA release (Germershaus et al. 2008). Further, Germeshaus et al. evaluated the cytotoxicity of these polymers and uptake and transfection efficiency of polyplexes in vitro. Under conditions found in cell culture, formation of aggregates and strongly decreased DNA condensation efficiency was observed in the case of chitosan polyplexes. These characteristics resulted in only 7% cellular uptake in NIH/3T3 cells and low transfection efficiencies in four different cell lines. By contrast, quaternization of chitosan strongly reduced aggregation tendency and pH dependency of DNA complexation. Accordingly, cellular uptake was increased 8.5-fold compared to chitosan polyplexes, resulting in up to 678-fold increased transfection efficiency in NIH/3T3 cells. Apart from reduction of the cytotoxicity, PEGylation led to improved colloidal stability of polyplexes and significantly increased cellular uptake compared to unmodified trimethyl chitosan. These improvements resulted in a significant, up to 10-fold increase of transfection efficiency in NIH/3T3, L929 and MeWo cells compared to trimethyl chitosan. This study not only highlights the importance of investigating polyplex stability under different pH values and ionic strength conditions but also elucidates correlations between physicochemical characteristics and biological efficacy of the studied polyplexes.

In an approach to target pDNA–chitosan complex using cell specific receptors, Mayu et al. employed mannose-modified chitosan (man-chitosan) to target macrophages expressing a mannose receptor (Hashimoto et al. 2006). The cellular uptake of pDNA–man-chitosan complexes through mannose recognition was then observed. The pDNA–man-chitosan complexes showed no significant cytotoxicity in mouse peritoneal macrophages, while pDNA–man-PEI complexes showed strong cytotoxicity. The pDNA–man-chitosan complexes showed much higher transfection efficiency than pDNA–chitosan complexes in mouse peritoneal macrophages. Observation with a confocal laser microscope suggested differences in the cellular uptake mechanism between pDNA–chitosan complexes and pDNA–man-chitosan complexes. Mannose receptor–mediated gene transfer thus enhances the transfection efficiency of pDNA–chitosan complexes.

Using a similar technique, Taku and his coworkers modified a chitosan derivative, 6-amino-6-deoxy chitosan (6ACT), by galactosylation (Satoh et al. 2007). A series of galactose-modified 6ACTs (Gal-6ACT) with degrees of substitution (d.s.) ranging from 3% to 50% per pyranose were prepared by reductive alkylation with lactose. DNA retardation assays showed that the electrostatic

interaction between Gal-6ACT and plasmid DNA was not changed by galactose modification up to 50% per pyranose of 6ACT. Gal-6ACT with a d.s. of 38% was bound to galactose-recognizing lectin, RCA120. A significant increase in transfection efficiency for HepG2 cells was observed at degree of substitutions ranging from 18% to 50% and at N/P values ranging from 1.5 to 2.5. Under optimum conditions, Gal-6ACT showed about 10 times higher efficiency than 6ACT. However, a slight uptake by the galactose receptors on hepatocytes was observed by flow cytometric analysis. Moreover, Gal-6ACT with a d.s. of 38% mediated efficient gene transfer into both A549 and HeLa cells lacking the galactose receptor. These results suggest that the enhancement of transfection efficiency of Gal-6ACT was not due to the increase of receptor-mediated cellular uptake. In addition, the enhanced gene transfer efficiency was not specific to the galactose modification because the efficiency of glucose-modified 6ACT for HepG2 cells was similar as that of Gal-6ACT.

Fascinated by the properties of chitosan, few researchers have explored the possibility of surface modification of poly(D,L-lactide-*co*-glycolide) (PLGA) nanosphere platform with chitosan for gene delivery by using the emulsion solvent diffusion (ESD) method (Tahara et al. 2008). By coating the PLGA nanospheres with chitosan, the loading efficiency of nucleic acid in the modified nanospheres was found to increase significantly. The release profile of nucleic acid from PLGA nanospheres exhibited sustained release after initial burst, while coating with chitosan reduced the initial burst of nucleic acid release and prolonged the drugs releasing at later stage.

Probing for a solution to track the efficiency of DNA delivery, Lee et al. (2008) employed fluorescence resonance energy transfer (FRET) to monitor the molecular dissociation of a chitosan–DNA complex with different molecular weights of chitosan. Chitosan with different molecular weights was complexed with plasmid DNA and the complex formation was monitored using dynamic light scattering and a gel retardation assay. Plasmid DNA and chitosan were separately labeled with quantum dots and Texas red, respectively, and the dissociation of the complex was subsequently monitored using confocal microscopy and fluorescence spectroscopy. As the chitosan molecular weight in the chitosan–DNA complex increased, the Texas red-labeled chitosan gradually lost FRET-induced fluorescence light when HEK293 cells incubated with chitosan–DNA complex were examined with confocal microscopy. This suggests that the dissociation of the chitosan–DNA complex was more significant in the high molecular weight chitosan–DNA complex. Fluorescence spectroscopy was also used to monitor the molecular dissociation of the chitosan–DNA complex at pH 7.4 and pH 5.0 and the results confirmed that the dissociation occurred in acidic environments. This finding suggests that the high-molecular-weight chitosan–DNA complex could easily be dissociated in lysosomes compared to a low-molecular-weight complex. Furthermore, the high-molecular-weight chitosan–DNA complex showed superior transfection efficiency in relation to the low-molecular-weight complex. Therefore, it could be concluded that the dissociation of the chitosan–DNA complex is a critical event in obtaining the high transfection efficiency of the gene carrier–DNA complex (Lee et al. 2008).

26.3 CLINICAL APPLICATIONS OF CHITOSAN–DNA SYSTEMS

To test the chitosan/DNA system as a potential DNA vaccine candidate, Kumar et al. (2002) used a strategy involving an intranasal gene transfer, referred to as IGT, complexed with chitosan–DNA nanospheres containing a cocktail of DNA encoding nine immunogenic syncytial virus (RSV) antigens. This system was tested against acute RSV infection in a BALB/c mouse model. The effectiveness and mechanism of this IGT strategy were investigated, and results demonstrated that IGT is safe and effective against RSV as well as it significantly attenuates pulmonary inflammation induced by RSV infection. A single dose of about 1 mg/kg body weight is capable of decreasing viral titers by two orders of magnitude (100-fold) on primary infection. This therapy works by induction of high levels of both serum IgG and mucosal IgA antibodies, generation of effective control response and elevated lung specific production of interferon (IFN)-γ with antiviral action. Also intranasal gene transfer (IGT) significantly decreases pulmonary inflammation and does not alter airway hyper responsiveness, making it safe for in vivo use.

Another application in using chitosan–DNA gene therapy is against Coxsackie virus B3 infections, which cause acute and chronic myocarditis (Xu et al. 2004). Intranasal delivery of chitosan–DNA complex prepared by vortexing DNA with chitosan resulted in transgenic DNA expression in mouse nasopharynx and also inducing mucosal SIgA secretion. Sun et al. (2004) have constructed a eukaryotic expression vector pVAX1-pZP3a as an oral ZP DNA contraceptive and successfully encapsulated in nanoparticles with Chitosan to target zona pellucida (ZP), the extra cellular matrix surrounding oocytes. After 5 days of feeding to mice, the transcription and expression of pZP3 was found in mouse alvine chorion. Okamoto et al. investigated the potential of chitosan in the form of inhaled powder for gene delivery purposes by preparing powders using pCMV-Luc as a reporter gene and a LM chitosan (3,000–30,000) as a cationic vector with supercritical CO_2. This powder was administered to the lungs of mice and their transfection efficiency was compared to that of DNA solution and DNA powder without the cationic vector. The gene powder with the cationic vector was found to be excellent gene delivery system to the lungs (Okamoto et al. 2003).

Bivas-Benita et al. (2004) has proposed chitosan–DNA nanoparticles for pulmonary delivery of a DNA vaccine for *Mycobacterium tuberculosis*. The authors used an HLA-A2 transgenic mouse model to investigate the effect of pulmonary delivery of a new pDNA encoding eight HLA-A*0201-restricted T cell epitopes from *M. tuberculosis* formulated in chitosan nanoparticles. Pulmonary administration of these nanoparticles was shown to induce the maturation of dendritic cells as well as induce increased levels of IFN-γ secretion compared to pulmonary delivery of plasmids in solution or the intramuscular immunization route (Bivas-Benita et al. 2004).

In another study, Khatri et al. (2008) investigated the preparation and in vivo efficacy of plasmid DNA-loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B. Chitosan pDNA nanoparticles were prepared using a complex coacervation process. Prepared nanoparticles were characterized for size, shape, surface charge, plasmid loading, and ability of nanoparticles to protect DNA against nuclease digestion and for their transfection efficacy. Nasal administration of nanoparticles resulted in serum anti-HBsAg titer that was less compared to that elicited by naked DNA and alum-adsorbed HBsAg, but the mice were seroprotective within 2 weeks and the immunoglobulin level was above the clinically protective level. However, intramuscular administration of naked DNA and alum-adsorbed HBsAg did not elicit sIgA titer in mucosal secretions that was induced by nasal immunization with chitosan nanoparticles. Similarly, cellular responses (cytokine levels) were poor in case of alum adsorbed HBsAg. Chitosan nanoparticles thus produced humoral (both systemic and mucosal) and cellular immune responses upon nasal administration. The study signifies the potential of chitosan nanoparticles as DNA vaccine carrier and adjuvant for effective immunization through noninvasive nasal route.

Although the conventional high-molecular-weight chitosans have a few drawbacks such as aggregated shapes, low solubility at neutral pH, and high viscosity at concentrations used for in vivo delivery and a slow onset of action (Zhou et al. 2008), the nonviral gene delivery systems based on chitosan are still regarded as one of the most efficient systems for DNA vaccine delivery. To circumvent the problems with high molecular weight and to improve the efficacy of DNA delivery, Zhou et al. (2008) examined the possibility of covalently attaching low-molecular-weight chitosans with an average molecular mass of 6 kDa (Chito6) to gold nanoparticles (GNPs). Further, the potency of the resulting Chito6-GNPs conjugates as vectors for the delivery of plasmid DNA was investigated both in vitro and in vivo. After delivery by intramuscular immunization in BALB/c mice, the Chito6-GNPs conjugates induced an enhanced serum antibody response 10 times more potent than naked DNA vaccine. Additionally, in contrast to naked DNA, the Chito6-GNPs conjugates induced potent cytotoxic T lymphocyte responses at a low dose (Zhou et al. 2008).

26.4 CHITOSAN–siRNA COMPLEX NANOPARTICLES

RNA interference (RNAi) has been discovered as a conserved mechanism in higher eukaryotic cells to eliminate harmful or unwanted genes, and thereby contribute to the defense from viral infection and generation of microRNA that regulate gene expression in many cellular pathways. Recently, small

interfering RNAs (siRNAs) have proven to be versatile agents for controlling gene expression in mammalian cells. They have also been shown more potent than conventional antisense strategies (Beale et al. 2003, Khan et al. 2004, Miyagishi et al. 2004). Overall, they appear to be a much more robust and efficient technology offering a significant potential. siRNA consisting of 21–23 nucleotides can regulate gene expression in mammalian cells through RNA interfering (RNAi). As the administration of siRNA could bypass nonspecific inhibition of protein synthesis induced by long double-stranded RNA (Sioud and Sorensen 2003, Sorensen et al. 2003), it has therefore been employed as a novel tool in blocking the expression of genes such as those expressed in infectious diseases and cancers. However, similar to hydrophilic and polyanion-mediated gene therapy, siRNA also suffers particular problems including poor cellular uptake, rapid degradation by ubiquitous nucleases as well as limited blood stability (Stein 1996, Urban-Klein et al. 2004). Because of these limitations, unassisted delivery of siRNA to the cells is frustrating. Although various chemical modifications of siRNA can be used to overcome these problems, these modifications possess disadvantages such as decreased mRNA hybridization, higher cytotoxicity, and increased unspecific effects (Weyermann et al. 2005). Therefore, effective systems that can protect and transport siRNA to the cytoplasm of the target cells are needed to exploit the promising potential applications offered by successful delivery of siRNA.

Chitosan can be used as a siRNA vector due to its advantages such as low toxicity, biodegradability, and biocompatibility (Illum 1998, Thanou et al. 2001). In an approach to develop chitosan nanoparticles for siRNA delivery, Katas and Alpar (2006), prepared chitosan nanoparticles by two methods of ionic cross-linking, simple complexation, and ionic gelatin using sodium tripolyphosphate (TPP). Both methods produced nanosized particles, less than 500 nm depending on type, molecular weight, as well as concentration of chitosan. In the case of ionic gelation, two further factors, namely chitosan to TPP weight ratio and pH, affected the particle size. In vitro studies in two types of cells lines, CHO K1 and HEK 293, have revealed that preparation method of siRNA association to the chitosan plays an important role on the silencing effect. Chitosan–TPP nanoparticles with entrapped siRNA are shown to be better vectors as siRNA delivery vehicles compared to chitosan–siRNA complexes possibly due to their high binding capacity and loading efficiency. This report suggests that chitosan–TPP nanoparticles show much potential as viable vector candidates for safer and cost-effective siRNA delivery.

Exploring the efficiency of chitosan/siRNA nanoparticles as a therapeutic agent, Liu et al. reports that the physicochemical properties (size, zeta potential, morphology, and complex stability) and in vitro gene silencing of chitosan–siRNA nanoparticles are strongly dependent on chitosan molecular weight (MW) and degree of deacetylation (DD) (Liu et al. 2007). High MW and DD chitosan resulted in the formation of discrete stable nanoparticles of 200 nm in size. Chitosan–siRNA formulations (N:P 50) prepared with low MW (10 kDa) showed almost no knockdown of endogenous enhanced green fluorescent protein (EGFP) in H1299 human lung carcinoma cells, whereas those prepared from higher MW (64.8–170 kDa) and DD (80%) showed greater gene silencing ranging between 45% and 65%. The highest gene silencing efficiency (80%) was achieved using chitosan–siRNA nanoparticles at N:P 150 using higher MW (114 and 170 kDa) and DD (84%) that correlated with the formation of stable nanoparticles of 200 nm. From their conclusions, it is evident that there is stillroom for improvement and for the optimization of gene silencing using chitosan–siRNA nanoparticles, the fine-tuning of the polymeric properties makes a huge difference.

26.5 CONCLUSIONS

In the field of gene therapy, the development of efficient and safe gene carrier systems that are capable to transferring DNA into cells is a major goal. Different systems have been developed in recent years, and chitosan is one of the most promising candidates among them. There are an enormous number of in vitro and in vivo studies, showing that chitosan is a suitable material for efficient nonviral gene therapy. In this review, we have summarized the recent advances in gene therapy, placing emphasis on the application of chitosan-conjugated DNA nanoparticles as novel nonviral vectors to improve the transfection efficiency.

Drawbacks with conventional high-molecular-weight chitosan prompted researchers to conduct studies using a series of chitosan oligomers, ranging in molecular weight from 1.2 to 10 kDa. By varying the molecular weight of chitosan, plasmid concentration and the stoichiometry of polymer–plasmid complex, the transfection efficiency, and DNA uptake can be tuned. These gene delivery systems based on chitosans can be conjugated with quantum dots for tracking and also be equipped with specific ligands for cell specific interaction. Together with gene therapy applications, chitosan nanoparticles contribute to the design of siRNA polyplexes for gene silencing and also as carriers of DNA vaccines, which will fuel clinical development pipelines in the near future.

All these studies highlighting the use of chitosan nanoparticles as biocompatible nonviral gene delivery systems present a platform for further optimization studies of chitosan-based gene delivery systems, for example, with regard to steric stabilization and cell specific targeting. Another promising area of research could be the matrix-based gene delivery using chitosan nanoparticles, which would find increasing utility and appeal for regenerative medicine. Studies with stem cells would be of particular interest as the encapsulated genes to drive the preferential differentiation of the seeded cells can further augment the micro environmental cues at the site of implantation.

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27 Chitinolytic Enzymes from the Moderately Thermophilic Bacterium *Ralstonia* sp. A-471: Characterization and Application

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CONTENTS

27.1 INTRODUCTION

Chitin, a β-1,4 polymer of *N*-acetyl-D-glucosamine (GlcNAc), is the second abundant biopolymer found in nature after cellulose (Muzzarelli 1999). This natural resource is relatively easily accessible, for example, as crab and shrimp shell waste. *N*-acetyl-chito-oligosaccharides and chito-oligosaccharides have varied biological functions and many potential applications in a wide range of fields (Tokoro et al. 1988, Hirano and Nagao 1989). To obtain enzymes that can be applied

to develop environment-friendly techniques in chitin-oligosaccharide production, we have isolated a thermophilic strain belonging to the genus *Ralstonia* (Sutrisno et al. 2004). Chitinase A was excreted into the culture medium and was constantly produced until the colloidal chitin was wholly degraded. The other chitinase, chitinase B, showed a trace amount of protein in the culture medium, and had weaker activity than *Ralstonia* chitinase A. We have isolated and purified chitinases A (Ra-ChiA) and B (Ra-ChiB) from *Ralstonia* sp. A-471. The enzymatic properties of these two enzymes were similar to each other (Ueda et al. 2005). To understand the genetic basis for the production of chitinases in *Ralstonia* sp. A-471, we attempted to clone and sequence the chitinase genes. In the cloning of the chitinase genes from *Ralstonia* sp. A-471, we got several positive clones and one of them indicated a homology to amino acid sequences of G-type lysozyme (Pooart et al. 2005) and *Clostridium* lysozyme-like enzyme (NCBI accession no. YP_001309935). This transformant had activities against several chitins and chitosans but not against the cell wall of *Micrococcus lysodeikticus* (Ueda et al. 2009). In this chapter, we review (1) the purification and characterization of chitinolytic enzymes from moderately thermophilic bacterium *Ralstonia* sp. A-471 and (2) cloning, sequencing, and the expression of G-type lysozyme gene with chitinase (Ra-ChiC) activity from the bacterium.

27.2 PURIFICATION AND CHARACTERIZATION OF CHITINOLYTIC ENZYMES FROM THE MODERATELY THERMOPHILIC BACTERIUM *RALSTONIA* SP. A-471

27.2.1 Enzyme Assay

Chitinase assay was determined by a modified Schales' procedure using colloidal and/or soluble chitin as the amount of enzyme that liberated 1 μmol of reducing sugar per minute (Imoto and Yagishita 1971). This method was used as the standard assay. To measure the activity toward other substrates, powdered chitin, colloidal chitin, carboxymethyl chitin, ethylene glycol chitin, chitosan 7B, chitosan 8B, chitosan 9B, chitosan 10B, *p*-nitrophenyl-β-D-*N*-acetyl glucosaminide (*p*NP-GlcNAc), and several chitin/chitosan oligomers were used instead of soluble chitin.

27.2.2 Purification and Characterization of Chitinases A and B

27.2.2.1 Cultivation and Purification of Chitinases (Chitinases A and B)

The cultivation and purification of *Ralstonia* sp. A-471 were described in the previous papers (Sutrisno et al. 2004, Ueda et al. 2005). It showed that chitinase A (Ra-ChiA) was the major enzyme and chitinase B (Ra-ChiB) was the minor enzyme.

27.2.2.2 Molecular Mass and N-Terminal Amino Acid Sequence

The molecular masses of Ra-ChiA and Ra-ChiB were estimated as 70 and 45 kDa by SDS-PAGE (Table 27.1) (Sutrisno et al. 2004, Ueda et al. 2005). The molecular mass of Ra-ChiA was almost the same as that of *Bacillus circulans* chitinase A1 (Bc-ChiA1), and that of Ra-ChiB was similar to those of *B. circulans* chitinase B1 (38 kDa), B2 (38 kDa), C (39 kDa), and D (52 kDa) (Watanabe et al. 1990). The N-terminal amino acid sequence of Ra-ChiA was determined to be ADPYLKVAYY-. This sequence was the identical to that of Ra-ChiB. These results indicate that Ra-ChiB was probably derived from Ra-ChiA, presumably by the proteolytic removal of an approximately 25 kDa polypeptide from the C-terminal region of the protein. The N-terminal amino acid sequences of Ra-ChiA and Ra-ChiB showed significant homology with those of chitinase A1 of Bc-ChiA1 (66% homology as amino acid identity) (Watanabe et al. 1990) and chitinase of *Bacillus halodulans* C-125 (80% homology as amino acid identity).

TABLE 27.1
Characterization of Ra-ChiA and Ra-ChiB

Characterization	Ra-ChiA	Ra-ChiB
Molecular mass	70 kDa	45 kDa
Optimum pH	5.0	5.0
pH stability	5.0–10.0	5.0–10.0
Optimum temperature	70°C	60°C
Thermostability	60°	60°C
Substrate specificity[a]	*N*-acetylated chitosans > CM-chitin > colloidal chitin	*N*-acetylated chitosans > CM-chitin > colloidal chitin
N-terminal amino acid sequence	ADPYLKVAYY–	ADPYLKVAYY–
GH family	Family 18	Family 18

[a] The specific activities of Ra-ChiB against chitins and *N*-acetylated chitosans were lower than those of Ra-ChiA.

27.2.2.3 Effects of pH and Temperature

The optimum pHs of Ra-ChiA and Ra-ChiB were determined to be 5.0 (Sutrisno et al. 2004, Ueda et al. 2005). The pH effects on the enzyme stabilities were analyzed by incubating the enzyme for 30 min at 37°C in several buffers. After incubation, the remaining activity was measured. The enzymes were stable over a pH range of 5–10. The optimum pHs of Ra-chiA and Ra-ChiB were the same as that of Bc-ChiA1. The enzymes were stable up to 60°C when incubating for 30 min. Ra-ChiA and Ra-ChiB are moderately thermostable enzymes, but Bc-ChiA is not stable against heat treatment.

27.2.2.4 Substrate Specificity

Both enzymes showed wide ranges of substrate specificities and were most active toward chitosan 7B (Sutrisno et al. 2004, Ueda et al. 2005). Among the substrates, their activities against insoluble substrate powdered chitin were lowest. The specific activities of Ra-ChiB against chitins and partially acetylated chitosans were lower than those of Ra-ChiA. In activity staining of chitinases from *Bacillus circulans* WL-12, the clear zone formed by chitinase A2 (BcChiA2) was less clear than that formed by Bc-ChiA1 (Watanabe et al. 1990). Zymogram analysis of the enzyme samples was done with a 10% SDS-PAGE containing 0.1% ethylene glycol chitin (Sutrisno et al. 2004). The clear zone of Ra-ChiB was also less clear than that of Ra-ChiA. Bc-ChiA1 has a chitin-binding domain in C-terminal region, and Bc-ChiA2 disrupted the chitin-binding domain. The properties of Ra-ChiB resembled those of Bc-ChiA2. Ra-ChiB might also be removed from the chitin-binding domain in the C-terminal region.

27.2.2.5 HPLC Analysis of Hydrolysis Products from (GlcNAc)2–6 and *N*-Acetylated Chitosans

HPLC analysis was carried out using TSK-Amide 80 column that can be used to separate α- and β-anomers of the hydrolysis products as well as to estimate the cleavage pattern of the enzyme (Arakane et al. 2000). In the early stage of the reaction, Ra-ChiA and Ra-ChiB produced the β-anomer of (GlcNAc)2 from the substrate (GlcNAc)6, whereas (GlcNAc)4 was almost equilibrium α- and β-anomers, indicating that the enzymes predominantly hydrolyze the second glycoside linkage from the nonreducing end of (GlcNAc)6 (Sutrisno et al. 2004, Ueda et al. 2005). *Ralstonia* chitinases were found to produce the β-anomer by hydrolyzing the glycosidic linkage, indicating that the enzymes are retained. The enzymes belong to the glycoside hydrolase family 18. The cleavage pattern and anomeric form of the hydrolysis product of *Ralstonia* chitinases were the same that of Bc-ChiA1. The transglycosylation product, of which the

polymerization degree was higher than that of initial substrate (GlcNAc)6, was found in the HPLC profiles. *Ralstonia* chitinases also resembled Bc-ChiA1 in its transglycosylation activity.

The hydrolysis of chitosan 7B, 8B, and 9B produced various oligosaccharides with degree of polymerization (DP) ranging from 1 to 8. The hydrolysis of chitosan 7B produced dimmer and hexamer as the major products; small amounts of monomer, heptamer, and octamer were also detected as minor products. The oligomer products are supposed to contain various GlcNAc/GlcN compositions or sequences that may have new biological functions. Furthermore, *Ralstonia* chitinases were highly active at acidic pH, which are beneficial since the solubility of partially *N*-acetylated chitosans increases at acidic pH values.

27.3 CLONING, SEQUENCING, AND EXPRESSION OF A NOVEL GOOSE-TYPE LYSOZYME GENE WITH CHITINASE (Ra-ChiC) ACTIVITY FROM THE MODERATELY THERMOPHILIC BACTERIUM *RALSTONIA* SP. A-471

27.3.1 Cloning and Sequencing of a Novel G-Type Lysozyme Gene

The plasmid coding Ra-ChiC was named pRa-C2 (Ueda et al. 2009). The 2.8 kb DNA fragment in the pRa-C2 was sequenced. An open reading frame (ORF) consisting of 756 bases starts at the ATG codon and ends with the stop codon TAT, encoding 251 amino acids. The molecular mass of the unprocessed protein was calculated to be 26,886. The protein derived from ORF of pRa-C2 appears to be a modular enzyme composed of a signal sequence, chitin-binding domain, linker, and lysozyme-like enzyme domain. The catalytic domain of Ra-ChiC showed significant homologies to those obtained from Rhea egg white (16.8%) and *Clostridium beijerinckii* (76.1%). Chitin-binding domain of Ra-ChiC had a significant similarity to that of *Bacillus circulans* ChiA1 (Watanabe et al. 1990). The molecular mass of catalytic domain of Ra-ChiC was 13 kDa, which was smaller than that of the G-type lysozyme (23 kDa). Thus, the *Ralstonia* enzyme was found to be an enzyme with a novel molecular construction. The active site of G-type lysozymes has a highly conserved Glu residue, which is believed to act as a general acid in catalysis (Glu73 in G-type lysozyme) (Grutter et al. 1983, Weaver et al. 1985, 1995). A Glu residue (Glu 141) was also conserved in Ra-ChiC. The discovery of Ra-ChiC raised the question of evolutionary relationships between the bacterial and goose egg enzymes. *Ralstonia* sp. A-471 studied in this article has been isolated from compost containing soil. *Ralstonia* sp. A-471 could possibly interact with animal eggs in soil, bringing about horizontal gene transfer from goose eggs to the *Ralstonia*. Ra-ChiC cannot hydrolyze the cell wall of *Micrococcus lysodeikticus*. It is possibly that the *Ralstonia* enzyme lost the lytic activity in the process of evolution.

27.3.2 Expression of a Novel G-Type Lysozyme Gene and Purification of the Recombinant Protein

The chitinase activity of the extract from *E. coli* JM 109 harboring pCRa-C2 was determined 0.363 U/mL culture (Ueda et al. 2009). The molecular mass of the recombinant enzyme was estimated to be 29 kDa from SDS-PAGE (Table 27.2). Ra-ChiC was obtained at 5.69 mg purified enzymes per liter of culture broth.

27.3.3 Characterization of a Recombinant Protein

27.3.3.1 Optimum pH and Temperature

The enzyme was active at pH 6.0 and 9.0 and stable over a pH range 5.0–10.0 (Ueda et al. 2009). This pH behavior was similar to those of the *Bombyx mori* chitinases and Yam chitinases (Koga et al. 1997, Arakane et al. 2000). The purified enzyme was showed maximum activity at 55°C and was stable up to 50°C when incubated for 30 min. It was indicated that Ra-ChiC is also the

TABLE 27.2
Comparison of Characterization of Ra-ChiC and G-Type Lysozyme

Characterization	Ra-ChiC[11]	G-Type Lysozyme[17]
Molecular mass	29 kDa	23 kDa
Optimum pH	6.0 and 9.0	6.0[a], 4.0[b]
pH stability	5.0–10.0	3.0–5.0
Optimum temperature	55°C	40°C
Thermostability	50°C	40°
Substrate specificity	Soluble chitin > *N*-acetylated chitosan > colloidal chitin, no activity against cell wall of *M. lysodeikticus*	—
Hydrolysis products from (GlcNAc)6	(GlcNAc)2+(GlcNAc)4 = (GlcNAc)3+(GlcNAc)3	(GlcNAc)3+(GlcNAc)3 > (GlcNAc)2+(GlcNAc)4
GH family	Family 23	Family 23

[a] Against *M. luteus*.
[b] Against (GlcNAc)5.

heat-stable enzyme. These results were similar to those of Ra-ChiA and Ra-ChiB (Sutrisno et al. 2004, Ueda et al. 2005).

27.3.3.2 Substrate Specificity

Among the substrates, the activity against insoluble substrate powdered chitin was the lowest (Ueda et al. 2009). Its specific activities toward chitosan 7B, 8B, and 9B were higher than that of colloidal chitin. Ra-ChiC was capable of degrading not only chitin but also chitosans with various degrees of acetylation. This property was also showed to Ra-ChiA and Ra-ChiB.

27.3.3.3 HPLC Analysis of Hydrolysis Products from (GlcNAc)2–6

Ra-ChiC was found to produce the α-anomer by hydrolyzing the β-1,4-glycosidic linkage, indicating that the enzyme is an inverter (Ueda et al. 2009). The G-type lysozymes (GH family 23) are also inverters. In the case of Ra-ChiC, almost the same concentration of (GlcNAc)4, (GlcNAc)3, and (GlcNAc)2 were obtained as the hydrolysis product of (GlcNAc)6 in the early stage of the reaction. Hence the (GlcNAc)6 splitting into (GlcNAc)3 +(GlcNAc)3 is predominant to that into (GlcNAc)2 + (GlcNAc)4 in G-type lysozymes (Honda and Fukamizo 1998, Pooart et al. 2005). The difference in splitting specificity between Ra-ChiC and the G-type lysozyme should be a result of the different subsite structures. Ra-ChiC and G-type lysozyme have different modular structures and differ in the size of the catalytic domain; such structural differences might cause a difference in the subsite structure. The enzyme showed high residual activity when incubated with organic compounds such as acetonitrile, methanol, and ethanol. From these results, the enzyme will be advantageous for practical applications. Further experiments are now underway to refine the substrate-binding cleft of this enzyme.

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28 Chitosan and Chitosan Derivatives as DNA and siRNA Carriers

Hu-Lin Jiang, Yun-Jaie Choi, Myung-Haing Cho, and Chong-Su Cho

CONTENTS

28.1 INTRODUCTION

Gene therapy is a powerful procedure in treating a variety of inherited and acquired diseases. Most of the gene therapy trials rely on viral vectors because viruses achieve a high efficiency of gene transfer *in vivo*. However, the use of viral vectors has several limitations such as immunogenicity, potential infectivity, complicated production, and inflammation of human body (Smith 1995). Nonviral vectors are rapidly receiving increased attention as gene delivery systems because of their biosafety, biocompatibility, and a high flexibility of size of the delivered nucleic acid (Li and Huang 2000). Among nonviral vectors, lipids and polymers are by far the most widely

used gene carriers as alternatives. Often used cationic lipids are Lipofectamine™ 2000 (Ciccarone et al. 1999), 1,2-dioleoyl-*sn*-glycero 3-phosphoethanolamine (DOPE) (Koltover et al. 1998), *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium ethyl sulfate (DOTAP) (Simoes et al. 2000), *N*-[1-2,3,-dioleyloxyl propyl]-*N*,*N*,*N*-trimethyl ammonium chloride (DOTMA), and 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) (Meidan et al. 2006). Generally, efficient lipoplex-mediated transfection was obtained *in vitro*, although lipoplex clears rapidly from the bloodstream (Liu and Huang 2002) and distributes widely in the body (Song and Liu 1998). Cationic synthetic polymers such as polyethylenimine (PEI) (Boussif et al. 1995), poly(L-lysine) (PLL) (Wu and Wu 1987), poly(L-histidine)-graft-PLL) (Benns et al. 2000), poly(amino ester) (PAE) (Anderson et al. 2005), polyanhydrides (Quick et al. 2004), and poly(amido amine) (Haensler and Szoka 1993) are extensively used as nonviral vectors. They display striking advantages, such as a selection of appropriate size, conjugation with specific targeting moieties, and chemical modification, whereas a drawback of cationic synthetic polymers as gene carriers is a limited knowledge regarding the complex formation with genes and their biological effects. Chitosan (Rolland 1998), protein (Ding et al. 1995), and dextran (Danna and Sompayrac 1981) are widely used as nonviral vectors in natural polymers. Among natural polymers, chitosan and chitosan derivatives have been used to be good gene carrier candidates because they are biocompatible and biodegradable, and have low immunogenicity and toxicity. However, the low transfection efficiency and low cell specificity of chitosan must be resolved for its use in clinical applications. The objective of this chapter is to summarize the use of chitosan and chitosan derivatives in gene therapy, and particularly several parameters affecting transfection efficiency or gene silencing in chitosan/gene complexes. The roles of chemical modification using cell-specific ligand and pH-sensitive groups for the enhancement of cell specificity and transfection efficiency *in vitro* and *in vivo* are also discussed. Furthermore, the role of chitosan as a carrier of small interfering RNA (siRNA) is described.

28.2 PARAMETERS AFFECTING TRANSFECTION EFFICIENCY OR GENE SILENCING OF CHITOSAN/GENE COMPLEXES

Chitosan is a linear cationic polysaccharide, composed of glucosamine and *N*-acetyl glucosamine units linked by β(1–4) glycosidic bonds. The chitosan is the most reported natural polymeric gene carrier, although the optimum conditions for transfection efficiency or gene silencing have not been clear. Therefore, several parameters such as the degree of deacetylation (DD) and molecular weight (MW) of chitosan, pH, serum, the charge ratio of chitosan to gene, and the cell type on the transfection efficiency or gene silencing will be explained.

28.2.1 Degree of Deacetylation

The DD of chitosan affects transfection efficiency or gene silencing because it influences charge density, solubility, crystallinity, and degradation (Aiba 1989). Chitosans with various DD were prepared by acetylation with acetic anhydride to evaluate their effect on the efficiency of gene transfection by Kiang et al. (2004). They reported that the decreased DD in chitosan resulted in a decrease of transfection efficiency in different cell lines such as HEK293, HeLa, and SW756 cells due to chitosan/DNA complexes destabilization in the presence of proteins, whereas intramuscular gene expression increased with DD over the time points tested, indicating that DD in chitosan is an important factor in gene transfection efficiency *in vitro* and *in vivo*. Liu et al. studied the effect of DD in chitosan on gene silencing (Liu et al. 2007). The results indicated that higher gene silencing was achieved using chitosan/siRNA complexes using higher (84%) than lower (45% and 65%) DD, because low DD has less charge interaction with siRNA and the complexes formed are unstable.

28.2.2 Molecular Weight

Generally, the biological and physicochemical properties of chitosan are closely related to its MW. Therefore, it can be expected that transfection efficiency or gene silencing promoted by chitosan will be affected by its MW. Huang et al. studied the effect of the MW of chitosan on transfection efficiency (Huang et al. 2005). The results indicated that low MW chitosan was less efficient at retaining DNA upon dilution, and, consequently, less capable of protecting DNA from degradation by DNase, and resulted in low transfection efficiency because chain entanglement contributes less to complex formation as the MW of chitosan decreases (Kiang et al. 2004). On the other hand, Lavertu et al. (2006) reported that maximum expression levels could be attained by adjusting the lowering MW and increasing DD or lowering DD and increasing MW. Also, Köping-Höggard et al. (2004) reported that chitosan oligomer (18 monomer units)/DNA complexes were shown to have higher transfection efficiency due to the better ability to release DNA from the complexes, although the heptamer of chitosan did not show any gene expression, suggesting that a good balance must be kept between extracellular DNA protection (better with high MW) versus efficient intracellular unpackaging (better with low MW) to obtain a high transfection efficiency or the gene silencing of chitosan (Köping-Höggard et al. 2004). Liu et al. (2007) reported that a higher MW of chitosan mediated efficient siRNA gene silencing than lower MW because longer-chain chitosan molecules may favor the hydrophobic interactions or hydrogen bonds between the sugar residues of chitosan and the organic bases of the nucleotide.

28.2.3 pH

The primary amines of chitosan become positively charged below pH 5.5 because the pK_a value of the chitosan is around 6.3–6.4 (Li et al. 1996). Therefore, it can be expected that the transfection efficiency of the chitosan/DNA complexes will be largely affected by the pH of the culture medium. Several researchers reported that the highest expression efficiency was obtained between pH 6.5 and pH 7.0 and the transfection efficiency rapidly decreased above pH 7.2 due to the dissociation of DNA from the chitosan/DNA complexes above pH 7.2 (Zhao et al. 2006).

28.2.4 Serum

The effect of serum on transfection efficiency is very important for the practical applications of gene therapy *in vivo* because transfection efficiency *in vivo* is inhibited by serum (Goldman et al. 1997). Sato et al. (2001) studied the effect of serum on the transfection efficiency of the complexes *in vitro*. The results showed that the transfection efficiency of the complexes was increased at up to 20% serum due to the increased cell function by the serum, and was decreased at 50% serum owing to cell damage, although PEI-mediated transfection was decreased at 10% serum (Erbacher et al. 1998).

28.2.5 Charge Ratio

Ishii et al. (2001) studied the effect of charge ratio on the transfection efficiency of the complexes. The results indicated that the transfection efficiency of the complexes was highest at the charge ratio of 3, although a lower MW and a lower DD chitosan requires a higher N/P ratio and a too high N/P ratio showed reduced transfection, an indication of an optimal range for the N/P ratio specific to the chitosan used (Lavertu et al. 2006).

28.2.6 Cell Type

Chitosan/DNA complexes have been reported to transfect various cell types such as HeLa, HEK293, A549, Caco-2, HT-1080, M69 mesenchymal cells, MG63, and COS-1 cells (Kiang et al. 2004).

Among the used cells, HEK293 cells were more efficiently transfected than other cells with chitosan/DNA complexes (Leong et al. 1998), although its exact mechanism is not clear.

28.3 SPECIFIC LIGAND MODIFICATION

For successful gene delivery, nonviral vectors have to be overcome at a number of extracellular as well as intracellular barriers until the carried DNA reaches its final destination, the nucleus. One strategy for overcoming the extracellular barriers in nonviral gene delivery is receptor-mediated endocytosis to enhance cellular uptake specifically.

The transfection efficiency of the complexes can be enhanced by a specific ligand modification of the chitosan owing to specific binding to the receptors of the cells with ligands.

28.3.1 Galactose Ligand

The asialoglycoprotein receptors (ASGPR) of hepatocytes were first identified by Pricer and Ashwell (1971), who found that circulating asialoglycoproteins (ASGPs) bind to, and are degraded by, hepatocytes. The hepatocytes possess cell surface receptors at a high density of 500,000 per cell (Ashwell and Harford 1982), which recognize and bind exposed galactose (Neufeld and Ashwell 1979).

Murata et al. (1997) first studied specific targeting to HepG2 cells using trimethylated chitosan (TMC) having antennary galactose residues. The modified chitosans had a tendency to increase the cellular recognition ability with an increase of galactose residues. Gao et al. (2003) also reported that the transfection efficiency was influenced by the galactosylation degree in the galactosylated chitosan with relatively low cytotoxicity. Jiang et al. (2007b) introduced galactose-bearing lactobionic acid to chitosan-graft-polyethylenimine (CHI-g-PEI) for hepatocyte specificity, as shown in Figure 28.1. As shown in Figure 28.2a, the GC-g-PEI/DNA complexes showed a much higher transfection efficiency compared to GC/DNA, CHI-g-PEI/DNA, and PEI/DNA complexes. Also, the GC-g-PEI/DNA complexes showed higher transfection efficiency compared to CHI-g-PEI/DNA complexes in HepG2 cells, but not in HeLa cells, which have no ASGPR on the cell surface, as shown in Figure 28.2b, suggesting the galactose ligand on GC-g-PEI-enhanced transfection efficiency in HepG2 cells due to the recognition of the ASGPR in the cells. Furthermore, the transfection of the GC-g-PEI was greatly inhibited in the presence of the galactose, as shown in Figure 28.2c, indicating the GC-g-PEI/DNA complexes were transfected by receptor-mediated endocytosis (Jiang et al. 2007). When *in vivo* distribution of ^{99m}Tc-labeled complexes was checked after i.p. injection in the mice, more ^{99m}Tc-GC-g-PEI/DNA complexes were accumulated in the liver after 180 min, whereas the accumulation of ^{99m}Tc-CHI-g-PEI/DNA complexes was reduced after 180 min, indicating that GC-g-PEI is more efficient in reaching the liver after i.p. administration (Jiang et al. 2007). Recently, Song et al. (2009) synthesized galactosylated chitosan (GC) by the introduction of galactose to the hydroxyl group in 6-position of the chitosan, although the GC/DNA complexes were transfected to HEK293 cells that do not have ASGPRs. Interestingly, Dong et al. (2008) reported that galactosylated low MW chitosan/oligodeoxynucleotide complexes had higher affinity to the macrophages than hepatocytes *in vitro*. And more complexes were taken up by Kupffer cells in liver than by parenchymal cells, suggesting that there might be other lectins involved in the endocytosis (Dong et al. 2008).

28.3.2 Mannose Ligand

Antigen-presenting cells (APCs) such as macrophages and immature dendritic cells (DCs) express high levels of mannose receptors used for the endocytosis and phagocytosis of a variety of antigens that expose mannose (Jiang et al. 1995). The APCs can activate the effector cells of the immune defense, the naïve T-cells and the B-cells, which are essential for the effective therapeutic manipulation of a wide spectrum of immune functions (Foged et al. 2002). Kim et al. (2006b) coupled mannose moieties with water-soluble chitosan (MC) to induce the receptor-mediated endocytosis for targeting

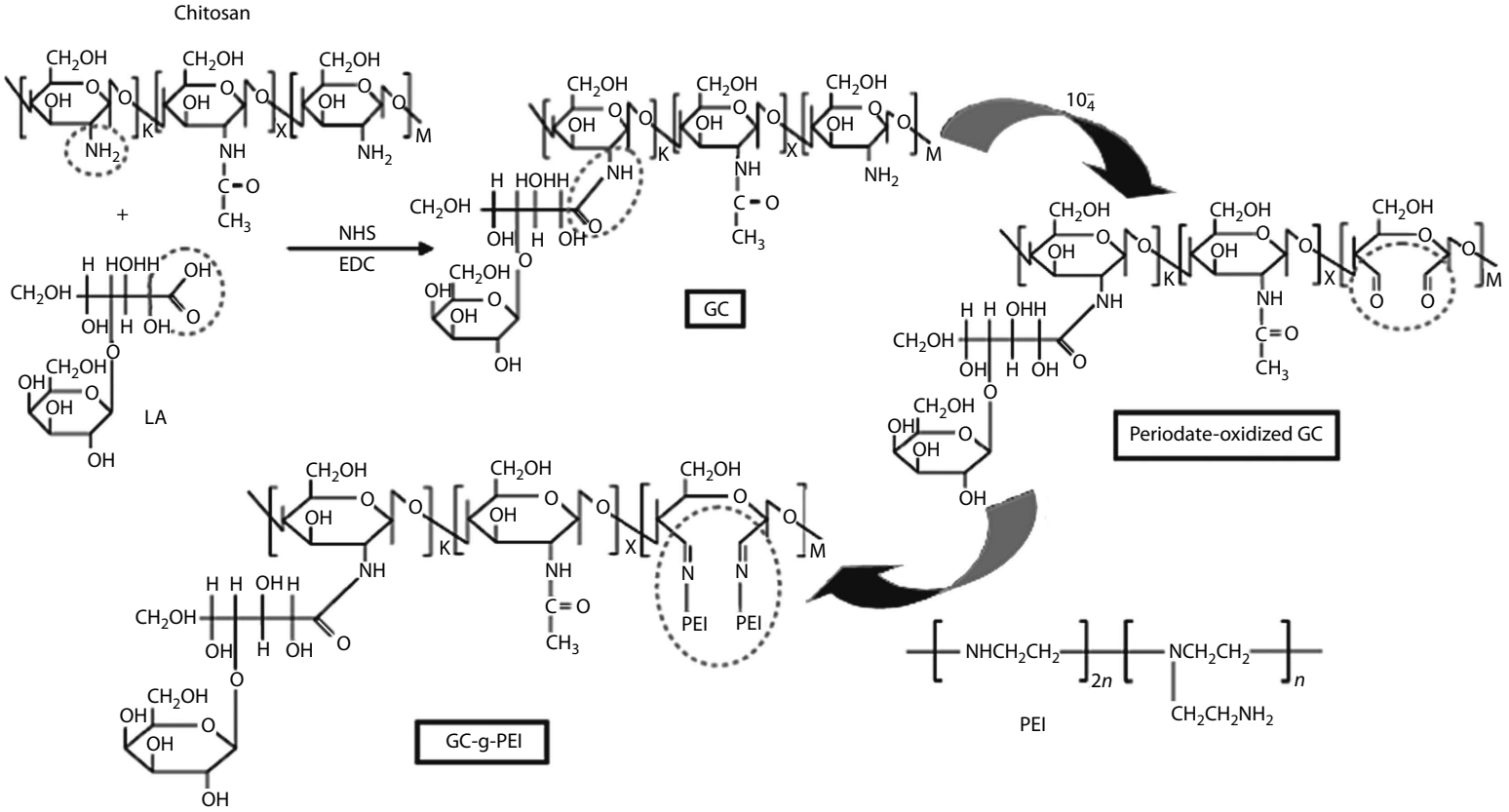

FIGURE 28.1 Proposed reaction scheme for synthesis of galactosylated chitosan-graft-polyethylenimine (GC-g-PEI). (From Jiang, H.L. et al., *Gene Ther.*, 14, 1389, 2007b. With permission.)

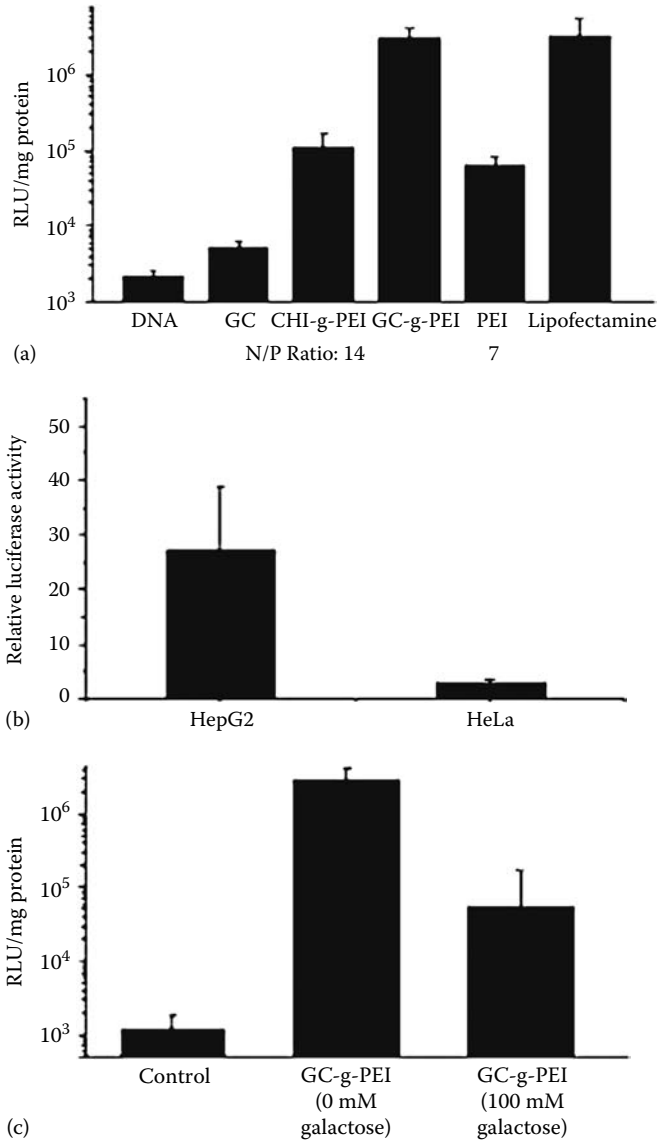

FIGURE 28.2 (a) Transfection efficiency of polymer/DNA (pGL3-control) complexes at functional N/P ratios in HepG2 cells, (b) luciferase activity of GC-g-PEI/DNA complexes at a charge ratio of 14 normalized by that of chitosan-graft-PEI (CHI-g-PEI)/DNA complexes on HepG2 and HeLa cells and (c) competition assay of GC-g-PEI/DNA complexes prepared at a charge ratio of 14 by adding free galactose (100 mM) as a competitor of the galactose in GC-g-PEI. (From Jiang, H.L. et al., *Gene Ther.*, 14, 1389, 2007b. With permission.)

Raw 264.7 macrophage cell line. The results indicated that MC/DNA complexes showed higher transfection efficiency than chitosan/DNA ones, and the transfection efficiency of the MC/DNA was decreased in the presence of mannose due to the receptor-mediated endocytosis mechanism (Kim et al. 2006). They also studied interleukin-12 (IL-12) gene delivery after an intratumoral injection of MC/plasmid encoding murine IL-12 complexes into BALB/c mice bearing tumor at the injected sites. The results indicated that an intratumoral delivery of MC/plasmid encoding murine IL-12 complexes suppressed more tumor growth than control and vector itself owing to the higher

production of IL-12 p70 and INF-y compared to control and vector (Kim et al. 2006a). Hashimoto et al. (2006) prepared mannosylated chitosan (MC) by the reductive ozonolyzation of allyl mannoside with chitosan by reductive alkylation in the presence of sodium cyanoborohydride. The mannose-modified chitosan/DNA complexes showed higher transfection efficiency than chitosan/DNA ones. However, Feng et al. reported that oligochitosan itself had receptor-mediated stimulatory effect in macrophages without mannose moieties as the specific ligand (Feng et al. 2004).

28.3.3 Folate Ligand

Folic acid (FA) has many unique properties such as a presumed lack of unlimited immunogenicity availability, functional stability, defined conjugation chemistry, and a favorable nondestructive cellular internalization pathway (Brzezinska et al. 2000). Also, the folate receptor (FR) binds to folic acid with high affinity (Kd < 1 nM) (Sudimack and Lee 2000). The FR is overexpressed in the various types of human cancers, whereas it is absent in most normal tissues (Zhao and Lee 2004). Therefore, the selective amplification of FR expression among human malignancies suggests its potential utility as targeted gene delivery. Mansouri et al. (2006) synthesized folate-coupled chitosan, and Chan et al. (2007) prepared folate-PEG-grafted chitosan for folate-mediated endocytosis although they did not perform transfection efficiency *in vitro*. Lee et al. (2006) covalently conjugated FA to chitosan to specifically deliver gene in human epithelial ovarian cancer OV 2008 cells and human breast cancer MCF-7 cells. The FA-conjugated chitosan exhibited significantly enhanced transfection efficiency in FR-overexpressing cancer cells compared to unmodified chitosan. Also, Fernandes et al. (2008) studied the protective effects of interleukin-1 receptor antagonist (IL-1 Ra) in bone metabolism *in vivo* using folate-chitosan. The folate-chitosan/DNA nanoparticles enhanced IL-1 Ra protein synthesis *in vitro* and showed a better protection against inflammation and abnormal bone metabolism *in vivo* compared to naked DNA and chitosan/DNA ones.

28.3.4 Transferrin Ligand

Transferrin as a ligand efficiently transfers small MW drugs, liposomes, and macromolecules through a receptor-mediated endocytosis mechanism (Deshpande et al. 1994) because the transferrin receptors are found on many mammalian cells (Dautry-Varsat 1986). Mao et al. (2001) coupled transferrin with chitosan nanoparticles prepared by chitosan and sodium sulfate. The transferrin-conjugated chitosan nanoparticles showed a fourfold increase of transfection efficiency in HEK293 cells and HeLa cells as compared with the chitosan ones.

28.4 pH-SENSITIVE MODIFICATION

There are several steps in gene transfection such as the DNA complexation, cellular uptake of the complexes, release of DNA or complexes from endosomes, release of DNA from the carriers, and transfer into the nucleus (Zabner et al. 1995). Among them, an inefficient release of DNA in the polymer/DNA complexes from endosomes into the cytoplasm is one of the primary causes of poor transfection efficiency as the intracellular barrier. Therefore, pH-sensitive chitosan carriers have been tried to overcome the intracellular barrier.

28.4.1 Urocanic Acid

Kim et al. (2003) coupled urocanic acid (UA) with water-soluble chitosan (UAC), as shown in Figure 28.3. The transfection efficiency in 293T cells by UAC/DNA complexes was increased with increasing the substitution value of UA due to the buffering capacity in the endosomal compartment, which is similar to that of PEI. Aerosol-containing UAC/programmed cell death protein

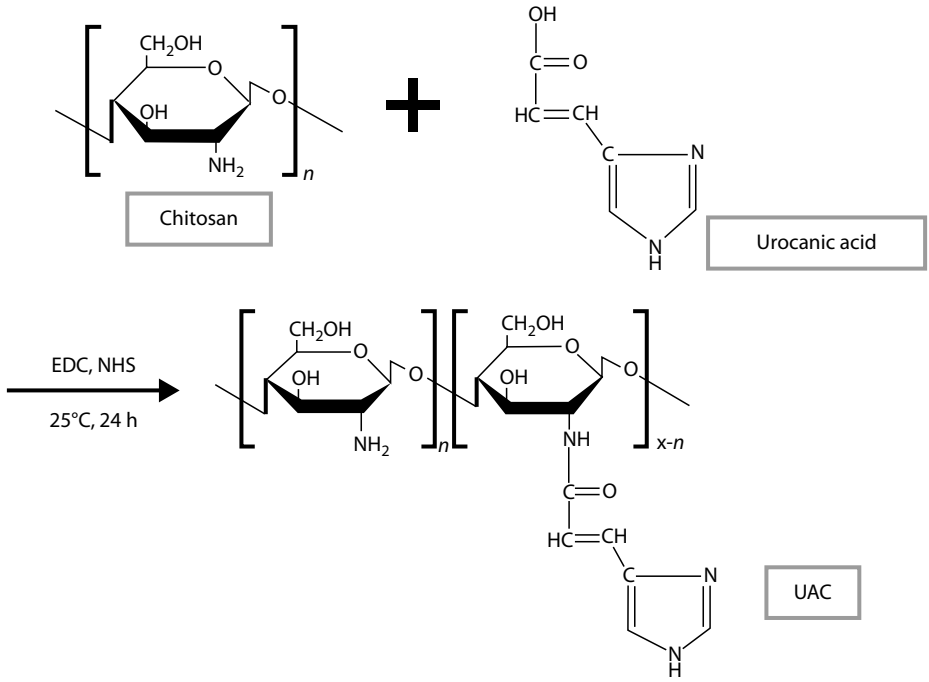

FIGURE 28.3 Synthetic scheme of UAC. (From Kim, T.H. et al., *J. Control. Release*, 93, 389, 2003. With permission.)

4 (PDCD4) complexes was delivered into K-ras null lung cancer model mice through the nose-only inhalation (Jin et al. 2006). The delivered UAC/PDCD4 complexes facilitated apoptosis, inhibited pathways important for cell proliferation, and efficiently suppressed pathways important for tumor angiogenesis. Also, the aerosols of UAC/phosphatase and tensin homolog deleted on chromosome 10 (PTEN) complexes were delivered into K-ras lung cancer model mice (Jin et al. 2008). The delivered PTEN suppressed lung tumor development significantly through nuclear complex formation between PTEN and p53, suppressing Akt-related signals as well as cell cycle arrest. Recently, wild-type p53 gene was transfected into HepG2 and into BALB/c nude mice through intratumoral injection using UAC by Wang et al. (2008a). The results indicated that UAC-mediated efficient p53 gene transfer-induced apoptosis by inhibiting the growth of HepG2 cells *in vitro* and *in vivo*. Ghosn et al. (2008) prepared imidazole acetic acid (IAA)-modified chitosan to introduce secondary and tertiary amines to the chitosan to improve its endosomal buffering and water solubility. The results indicated that up to 100-fold increase in the transfection efficiency of DNA was obtained for IAA-modified chitosan as compared to unmodified chitosan, nearly matching that of PEI 25K.

28.4.2 PEI

PEI is one of the most successful and widely used nonviral carriers because it has an excellent buffering capacity toward endosomal environment by proton sponge effect and thus facilitates endosomal escape to the cytoplasm. On the other hand, the PEI has high cytotoxicity in many cell lines, although the cytotoxicity of PEI depends on its MW.

Wong et al. (2006) synthesized PEI-graft-chitosan by the cationic polymerization of aziridine in the presence of water-soluble oligo-chitosan to get a combination of PEI having a proton sponge effect and chitosan having biocompatibility. The PEI-graft-chitosan showed higher transfection efficiency than that of PEI 25K *in vitro* and *in vivo* with low cytotoxicity. Jiang et al. (2007) prepared chitosan-graft-PEI by an imine reaction between the periodate-oxidized chitosan and low MW of PEI (1800 Da), as

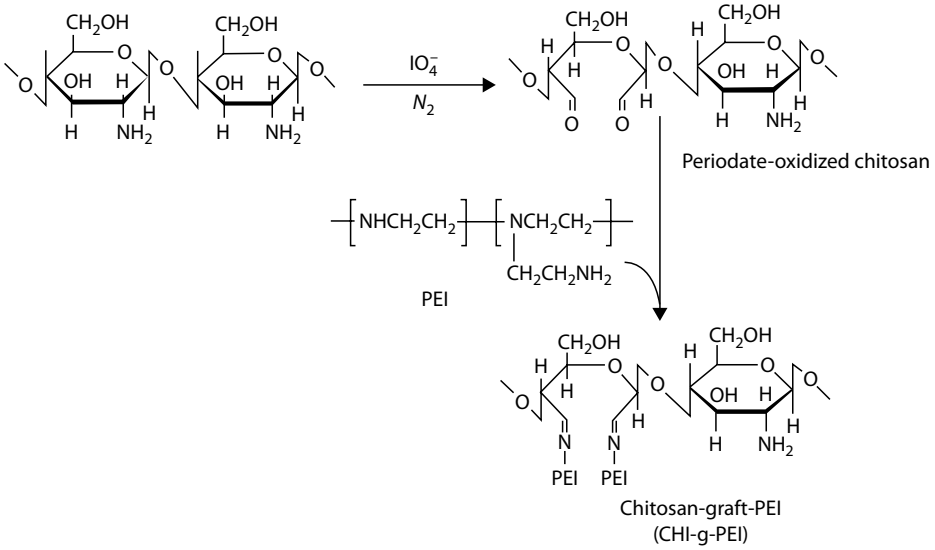

FIGURE 28.4 Proposed reaction scheme for synthesis of CHI-g-PEI. (From Jiang, H.L. et al., *J. Control. Release*, 117, 273, 2007a. With permission.)

shown in Figure 28.4. The chitosan-graft-PEI/DNA complexes showed higher transfection efficiency than PEI 25K in HeLa, 293T, and HepG2 cell lines with low cytotoxicity due to the buffering effect of PEI. Lu et al. (2008) grafted a low MW of PEI (800 Da) to *N*-maleated chitosans (NMC-g-PEI) by Michael addition reaction. The NMC-g-PEI copolymers showed high transfection efficiency, comparable to PEI 25K in 293T and HeLa cells with low cytotoxicity except for NMC 50K-g-PEI, although the particle sizes of the NMC-g-PEI/DNA complexes were 200–400 nm. Recently, Lou et al. (2009) prepared PEI-graft-chitosan using ethylene glycol diglycidyl ether as a spacer to improve the water solubility as well as the inherent transfection efficiency of chitosan. The results indicated that the transfection efficiency of PEI-g-chitosan/DNA complexes were significantly higher than chitosan/DNA ones at the weight ratios higher than 2.5 with low cytotoxicity.

28.5 siRNA DELIVERY

The discovery of RNA interference (RNAi)-mediated specific gene silencing has substantially increased our knowledge of the molecular mechanisms involved in the development of cancer during the last decade (Jere et al. 2009). In RNAi, double-stranded RNA molecules are cleaved by cellular enzyme "Dicer" to generate the short/small interfering RNA (siRNA) of 21–25 nucleotides that then guide the RNAi-induced silencing complex (RISC) to cleave target mRNAs that share sequence identity with siRNA. However, the efficient delivery of synthetic siRNA is still a major bottleneck in its success. In this section, the state of siRNA delivery using chitosan *in vitro* and *in vivo* will be covered.

28.5.1 *In Vitro* Application

Zhang et al. (2005) firstly used chitosan nanoparticles, Nanogene 042, to deliver plasmid-born siRNA targeting the NS1 gene (siNS1) to examine the potential and mechanism of siNS1 to inhibit respiratory syncytial virus (RSV) replication in A549 cells. They found efficient silencing of NS1 gene that attenuated RSV infection with gene-specific fashion using Nanogene 042.

Howard and Kjems (2006) reported that chitosan/siRNA complexes effectively silenced EGFP expression in both H1299 human lung carcinoma cells (77.9% reduction) and murine peritoneal macrophages (89.3% reduction). Katas and Alpar (2006) reported that preparation methods such as

the simple complexation and ionic gelation of siRNA association to the chitosan played an important role on the silencing effect in CHO K1 and HEK 293 cells. Chitosan-sodium tripolyphosphate (TPP) nanoparticles with entrapped siRNA showed better silencing effect than chitosan/siRNA complexes possibly due to their high binding capacity and loading efficiency. Liu et al. (2007) studied the effects of chitosan properties such as MW, DD, and N/P ratio on gene-silencing efficiency. The results indicated that higher MW, higher DD, and high N/P ratio are vital requirements for efficient siRNA-mediated gene silencing. Also, they reported that siRNA binds to chitosan in a different manner from that observed with DNA because the shorter length and linearity of siRNA is a prime difference. Tan et al. (2007) prepared chitosan nanoparticles encapsulated with flurorescent quantum dots (QD) as a self-tracking and transfection agent to deliver human epidermal growth factor receptor-2 (HER2) siRNA in SKBR3 cells. The results showed that the targeted delivery of HER2 siRNA to HER2-overexpressing SKBR3 breast cancer cells was specific to the chitosan/QD nanoparticles labeled with HER2 antibody and this system can be used for the long-term tracking of siRNA into cells due to the superior optical properties of the QD. Andersen et al. (2008) checked the specific and efficient knockdown of EGFP in H1299 human lung carcinoma cells transfected in plates precoated with chitosan/siRNA formulation to remove the necessity for both siRNA reconstitution immediately prior to use and adding onto cells. The results indicated that the silencing of the proinflammatory cytokine tumor necrosis factor was observed in the RAW macrophage cell line by the lyophilized chitosan/siRNA complexes, suggesting that the coating can improve the biocompatibility. Duan et al. (2008) synthesized CS-g-(PEI-b-mPEG) copolymer to deliver siRNA-targeting IkappaB kinase subunit beta (IKKbeta) into human Tenon's capsule fibroblasts (HTFs) to inhibit the proliferation of HTFs. The results indicated that the expression of IKKbeta was downregulated at both the mRNA and protein levels, and the proliferation of HTFs *in vitro* was effectively suppressed through the blocking of the NF-kappaB pathway. Similarly, Jere et al. (under submission) studied the silencing of Akt 1 expression in A549 cells using CHI-g-PEI/Akt 1 siRNA complexes. The results proved the enhanced efficiency of the CHI-g-PEI over the PEI 25K in the Akt 1 siRNA-mediated silencing of Akt 1 expression resulting in the inhibition of Akt 1-mediated signaling pathway. Recently, Wang et al. (2009) used chitosan-TPP nanoparticles to deliver TGFB 1 shRNA to silence TGFB 1 expression in rhabdomyosarcoma cells. The results indicated that the knockdown of TGFB 1 by shRNA/chitosan-TPP resulted in a decrease in cell growth *in vitro* and tumorigenicity in nude mice.

28.5.2 *In Vivo* Application

Only a few researches are available explaining the *in vivo* application of chitosan/siRNA complexes in silencing gene expression owing to the initial stage of this area. Among them, the inhibition of RSV infection with intranasal siRNA/chitosan nanoparticles targeting the viral NS 1 gene was the first trial *in vivo* (Zhang et al. 2005). The results indicated that mice treated intranasally with siNS1/chitosan nanoparticles after injection with RSV showed substantially decreased virus tiers in the lung and decreased inflammation compared to control. Howard and Kjems (2006) used chitosan/siRNA complexes to silence gene expression in transgenic EGFP mouse model through nasal administration. The results showed an effective knockdown of EGFP without showing any adverse effects. Pille et al. (2006) studied the efficient and safe intravenous administration of encapsulated anti-Rho A siRNA in chitosan-coated poly(isohexyl cyanoacrylate)(PIHCA) nanoparticles. The intravenous administration of chitosan-coated PIHCA/anti-Rho A siRNA nanoparticles significantly reduced cancer aggressivity *in vivo* by 90% through the knockdown of overexpressed Rho A in the cancer cells. Similarly, de Martimprey et al. (2008) used chitosan-coated PIHCA to deliver ret/PTC1 siRNA to inhibit a tumor growth of mice after intratumoral administration. Kong et al. (2007) reported that an intranasal administration of chitosan/siNS1 nanoparticles in rats before RSV exposure was effective in reducing virus titers in the lung and in preventing the inflammation compared to the control group. Wang et al. (2008b) delivered siRNA for natriuretic peptide receptor A

(NPRA) using imiquimod cream mixed with chitosan to the skin of asthma model mice. The results indicated that BALB/c mice treated with imiquimod cream containing siNPRA/chitosan nanoparticles showed significantly reduced airway hyper-responsiveness and pro-inflammatory cytokines IL-4 and IL-5 in lung homogenates compared to controls. Recently, Howard et al. (2009) reported that chitosan/siRNA nanoparticles mediated TNF-alpha knockdown in systemic macrophages by intraperitoneal administration in a murine arthritis model mice and resulted in the reduction of local and systemic inflammation.

The finding of efficient siRNA carriers will have an enormous impact on the biological researches and clinical potentials in various diseases.

28.6 CONCLUSIONS

As described in this chapter, among nonviral vectors, chitosan and chitosan derivatives have been developed for DNA and siRNA delivery systems. The transfection efficiency or gene silencing depended on the degree of deacetylation and MW of the chitosan, pH, serum, charge ratio of chitosan to gene, and cell type. A number of *in vitro* and *in vivo* studies have indicated that they are suitable materials as nonviral carriers. However, the continued development of the structure–function relationships and fundamental studies of cellular processes *in vitro* and *in vivo* should be performed for the future direction of chitosan as a gene carrier. Also, more preclinical studies need to be carried out for approval in clinical trials and commercialization as a gene carrier because the majority of studies carried out so far are only limited *in vitro* and in animal models. Furthermore, siRNA delivery using chitosan and chitosan derivatives may promote a new era of therapeutics for the cure of grave diseases.

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29 Metabolic Pathway of Chitin and Its Oligosaccharides in Marine Bacterium Vibrios

Jae Kweon Park

CONTENTS

29.1 INTRODUCTION

The vast amount of chitin that is produced annually is virtually beyond one's ability to conceptualize and yet only traces are present in marine sediments. Although other organisms are capable of degrading and digesting chitin (Bassler et al. 1991b; Kawachi et al. 2001; Lan et al. 2006; Zhu et al. 2007; Simunek et al. 2008; Wang et al. 2008a), which is a highly insoluble linear polysaccharide, it appears that the large quantity of this insoluble polymer is rapidly turned over by marine bacteria, at least in marine ecosystems (Bassler et al. 1989; Bassler et al. 1991a). Earlier studies demonstrated the significant role of genes encoding some specific enzymes involved in the chitin catabolic pathway; however, the emerging picture shows that chitin catabolic mechanism follows a highly sophisticated and complex series of coordinated processes (Meadow et al. 1987; Yu et al. 1987, 1993; Keyhani and Roseman 1996a; Keyhani et al. 1996, 2000a; Park et al. 2000, 2002a,b; Meibom et al. 2004; Byung-Ok Jung and Park 2008). Although different genes and their protein products with potential functions in chitin and chitosan degradation and their catabolic pathways have been well studied, much deeper understanding is required to delineate the exact pathways of the chitin and chitosan catabolic processes in marine bacterium Vibrios and other chitosanolytic organisms. This chapter describes biologically functional enzymes involved in the chitin catabolic pathway in marine bacterium Vibrios.

29.1.1 Chitin and Chitosan

Chitin, following the most abundant natural linear homopolymer cellulose that is widespread in most biospheres, is a highly insoluble aminopolysaccharide composed of β-(1,4)-linked

N-acetylglucosamine residues (GlcNAc). Evidently, chitin differs structurally from cellulose in the substitution of acetamide groups at the C-2 position in place of hydroxyl groups. Chitin can be found as an primary structural component in the hard outer shall of shrimps, crabs, and many other organisms including crustaceans and arthropods, and is also found as major component in the cell walls of many fungi as a complex matrix with other carbohydrates and polypeptide and glucans (Austin et al. 1981; Horst 1990; Rebers and Willis 2001; Merzendorfer 2006; Matsuda et al. 2007; Di Mario et al. 2008; Hayes et al. 2008). In particular, chitin cannot be dissolved in water but is relatively stable under mild acidic or basic conditions. Because of the insolubility of chitin, fewer applications of its biological properties have been studied compared to chitosan, which is a deacetylated form of chitin. Of particular interest is carboxymethyl chitin, a derivative of chitin prepared by carboxymethylation reaction. It can be soluble in water and has versatile applications in various fields as drug carrier in biomedical science and cosmetic industries (Tokura et al. 1987; Watanabe et al. 1990; Mobed and Chang 1991; Tokura and Tamura 2001; Lazarova et al. 2006; Uda et al. 2006; Kariya et al. 2007; Mezzana 2008).

Chitosans, the partially or fully deacetylated chitin molecules (Alves and Mano 2008; Hayes et al. 2008), are a family of linear binary heteropolysaccharides containing a mixture of both D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine monomers (GlcNAc) that are joined by β-(1–4) linkages. Cationic heteropolysaccharides tend to have degrees of *N*-acetylation ranging from 5% to 30% (Zamani et al. 2008) and have large adsorption area used as a gel-like dispersion in diluted acid. The relative amount of both residues and degrees of polymerization of chitosan can influence the properties of the amphophilic polymers and their application potential (Pelletier et al. 1990; Ng et al. 2006). The chemical structure of chitosan is characterized by the relative content ratio between the two monomer residues GlcN and GlcNAc and their distributions among the chains. Also chain length of chitosan is an important parameter to determine its biological function. The chemical composition of chitosan is routinely evaluated by ^{1}H-NMR, however, the distribution of GlcN and GlcNAc and the length of chains are yet to be considered, because the different chemical compositions of chitosan will be significantly reflected in their biological properties such as solubility, hydrophobicity, charge density, and susceptibility to the degradation (Kittur et al. 2003; Einbu and Varum 2007; Alves and Mano 2008; Lillo et al. 2008; Kasaai 2009). Over several decades, chitosan has a number of applications in various fields of waste water treatment, food sciences (Huang et al. 2005), pharmaceuticals (Lee et al. 2006; Cardenas et al. 2008), nutraceuticals (Anraku et al. 2009), cosmetics (Je and Kim 2006), and biomedical sciences including gene delivery using these fundamental properties (Ylitalo et al. 2002; Ishihara et al. 2003; Krauland et al. 2006; Jordan et al. 2007). However, in order to verify their properties as bioactive materials in many applications, chemical composition and chain length should be carefully studied to optimize the application efficiency of chitin and chitosan, oligosaccharides and their derivatives.

29.1.2 Glucosamine and *N*-Acetylglucosamine

Both amino-monosaccharides glucosamine (GlcN) and its deacetylated form *N*-acetyl-glucosamine (GlcNAc) are implicated as precursors for the biosynthesis of polymers and proteoglycans and cell walls of many organisms, as known as widely spread in marine organism especially found as a major component in the exoskeletons of crustaceans and other arthropods. These amino-monosaccharides can be obtained by chemical or enzymatical hydrolysis of chitin by combination of relative *endo*- and *exo*-type enzymes such as chitinases, chitobiases, and β-*N*-acetylglucosaminidase, as well as chitosan with chitosanases and glucosaminidases, respectively (Roseman 1957; Park et al. 1999; Tanaka et al. 2004; Makino et al. 2006; Konieczna-Molenda et al. 2008; Wang et al. 2008a). Especially, glucosamine has an excellent safety profile and has been found to provide significant benefits in several clinical disorders. For instance, glucosamine stimulates the biosynthesis of hyaluronic acid, then this results in accelerating wound healing, improving skin hydration, and

decreasing wrinkles. It is worthy to note that adequate amounts of glucosamine in the blood are necessary for the production of hyaluronic acid known as to be one of the essential substances to heal skin injuries (Roberts and Harding 1994; McCarty 1996; MacKay and Miller 2003; Bissett 2006). Furthermore, both chitin and chitosan oligosaccharides with several residues in chains produced by partial chemical or enzymatical hydrolysis have been applied in various fields of study due to their specific potential functions such as antibacterial (Lillo et al. 2008), antifungal, and anticancer activities (Wang et al. 2008b). For instance, chitooligosaccharides were also known as a bioactive material against *Candida albicans* infection that appears that GlcNAc metabolism of *C. albicans* is closely associated with its virulence (Yamada-Okabe et al. 2001; Sengupta and Datta 2003) and increased immune activities of T-lymphocytes, macrophages, and natural killer cells in tumor-bearing mice (Madan et al. 1997; Esteban et al. 2000, 2001; Shibata et al. 2001; Krenek et al. 2007).

29.2 BIOLOGICAL DEGRADATION OF CHITIN AND CHITOSAN

Chitin is a naturally abundant amino polysaccharide and so are the enzymes able to degrade this polysaccharide and its partially and/or fully deacetylated derivatives (i.e., chitosan). In general, chitin-containing organisms need some kind of chitinolytic enzymes for growth. Whereas, some microorganisms produce functionally different types of chitinolytic or chitosanolytic enzymes to exploit them as fundamental sources of energy and nitrogen (Roseman 1957; Yu et al. 1993; Kittur et al. 2003; Lan et al. 2006; Li et al. 2007). These enzymes are produced not only by chitin-containing organisms, but they can also be produced by other organisms as part of their defense against chitin-containing pathogens (Fukamizo 2000; Mayer et al. 2002). Enzymes that are able to hydrolyze glycosidic bonds are designated as glycosidases and are currently classified into more than 80 families. Although, catalytic mechanisms of glycosidases are well studied, chitinases and chitosanases differ in terms of their substrate specificity and preferences for the degree of acetylation and their sequence in polymer chains (Fukamizo 2000; Tanaka et al. 2003, 2004; Trombotto et al. 2008). For instance, family 18 chitinases, by far the most widespread and best studied, vary from bacteria to man, preferably cleave the linkage of GlcNAc–GlcNAc and GlcNAc and GlcN sequences, whereas family 19 chitinases preferably hydrolyze the linkage of GlcNAc–GlcNAc and GlcN–GlcNAc sequences of chitin. On the other hand, many chitosanases have shown substrate specificity against GlcN–GlcN and/or GlcN–GlcNAc and/or GlcNAc–GlcN in the deacetylated form of chitin. Therefore, both enzymes, chitinase and chitosanase, are basically distinguished by their substrate specificity.

29.2.1 Diversity of Chitinases and Chitosanases

Chitinase (EC3.2.1.14) and chitosanases catalyze the degradation of chitin and chitosan into smaller sized oligosaccharides $(GlcNAc/GlcN)_n$, $n = 2–10$, which have been implicated as potentially functional bioactive materials in many applications, as described above. Over several decades, chitinolytic and chitosanolytic enzymes have been purified and characterized from many organisms such as *Aeromonas* sp. (Lan et al. 2006), *Serratia* sp., *Matsuebacter* sp. (Park et al. 1999), *Streptomyces* sp. (Tremblay et al. 2001), and *Vibrio* sp. (Bassler et al. 1991a,b) in nature. Especially *Sphingomonas* sp. CJ-5 (Zhu et al. 2007) and *Pseudomonas* sp. (Wang et al. 2008a) have been identified as having both chitinolytic and chitosanolytic enzymes. Different organisms produce various kinds of chitinolytic enzymes for different purposes based on their own biology and physiology (Simunek et al. 2008; Shuhui et al. 2009). For instance, plant chitinases are induced by many pathogens and play an important role in defense mechanisms (Punja and Zhang 1993; Benhamou 1995; Kirubakaran and Sakthivel 2007). In addition, chitinases are also involved in the diversity of biological functions such as modification of ice crystal growth of plants, when exposed to cold in winter, plants produce chitinase-like antifreezing protein (AFP) (Pihakaski-Maunsbach et al. 2001). Most recently,

Park et al. (2008) demonstrated that photosynthetic green alga enable chitinolytic enzyme production in the presence of colloidal chitin used as the sole carbon source in culture medium under specified conditions.

The enzymatic hydrolysis of chitin and its catabolic mechanisms have been studied for almost a century. Thus far, much less is known about the metabolic pathway of chitosan, although many kinds of chitosanolytic enzymes from various organisms have been identified and their tertiary structure of proteins has been determined (Tremblay et al. 2000). Instead many studies demonstrated the substrate specificity and catalytic site of enzymes to engineer their function with the great benefit of producing different sequence of oligosaccharides, which can be obtained from the combination of different enzyme activities. To do so, genetic engineering of sequence encoding chitosanolytic enzymes has been considered the primary key point to modify the biological function of enzymes that enable to produce mono-oligomers and/or hetero-oligomers with high potential activities including antitumor (Wang et al. 2008b), antioxidant (Chen et al. 2003; Anandan et al. 2004; Guo et al. 2005; Xing et al. 2005; Koryagin et al. 2006; Sun et al. 2008; Anraku et al. 2009), and immune enhancing effects (Esteban et al. 2001). Therefore, these findings collectively led to the emergence of many potential opportunities to find out novel function for products and industrial value for specific enzymes with high substrate specificity for each end product. It would be tightly regulated by the degree of acetylation and/or deacetylation of chitin, sequence of chain and its length, and the diversity of organisms expressing relative enzymes using these materials as substrate or precursor. Therefore, further studies are absolutely necessary to understand signaling transduction in metabolic pathway of chitin and chitosan in well-studied organisms prior to seeking their further applications in human lives.

29.2.2 Chitin Catabolic Cascade in Marine Bacterium

Here, we describe the chitin catabolic cascade in marine bacteria, namely, *Vibrio* species. Over several decades, various chitinases from many different organisms have been purified for various applications under different conditions. These enzymes have been found from many organisms as described above. Consequently, chitin is thought to be one of most important organic sources in marine ecosystems, with several million tons produced annually in the marine biosphere alone. Many earlier works demonstrated that marine bacteria were preliminary responsible for this massive turnover into marine ecosystems, and play a critical role in converting this insoluble aminopolysaccharides into smaller size of oligosaccharides that might be utilized as a carbon and nitrogen source in chitinolytic microorganisms and many other marine organisms in oceans as well (Wortman et al. 1986; Baty et al. 2000; Howard et al. 2003; Li and Roseman 2004; Bhowmick et al. 2007). Especially, it has been previously shown that the chitin catabolism cascade in marine bacterium *Vibrio furnissii* and *Vibrio cholera* comprises very complex signaling transduction and many biologically functional proteins involved in the metabolic pathways. There are at least two different types of enzymes that are required to hydrolyze chitin substances and utilize their end products as carbon and/or nitrogen sources in growth including a transport system (Bouma and Roseman 1996; Keyhani et al. 1996). An extracellular chitinase known as *endo*-type that mainly yielded the disaccharide *N,N′*-diacetylchitobiose, $(GlcNAc)_2$ and/or $(GlcNAc)_3$, and an intracellular and/or extracellular "chitobiase," or β-*N*-acetylglucosaminidase (Bassler et al. 1991a; Chitlaru and Roseman 1996) as an *exo*-type enzyme, which gives the end product GlcNAc from chitooligosaccharides $(GlcNAc)_n$, n = 2–6. However, the chitin catabolic processing cascade even in marine bacteria is complex as described, more than previously expected.

29.2.3 Role of Marine Bacteria in Chitin Degradation

Many earlier work demonstrated that marine bacteria such as *V. cholerae* and/or *V. furnissii* play a critical role in converting of chitin to chitooligosaccharides $(GlcNAc)_n$, n = 2–6, and were primarily

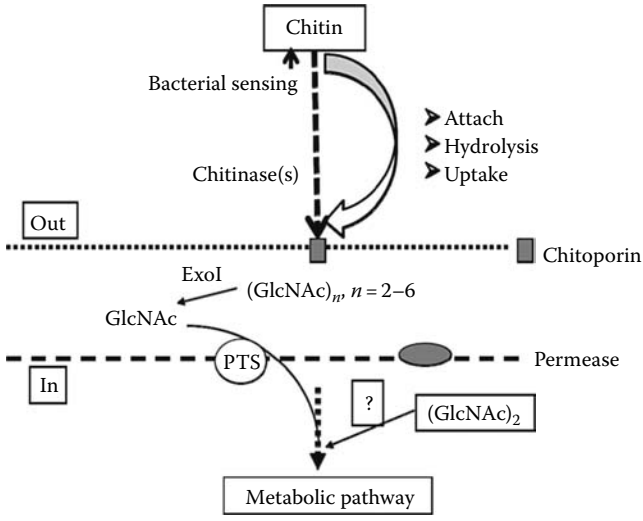


FIGURE 29.1 Schematic flow of chitin degradation in marine bacterium, Vibrios. Immense quantities of chitin are degraded by marine bacteria, and this process involves at least three steps: (1) bacterial chemotaxis to extracellular polysaccharide chitin; (2) hydrolysis and uptake of soluble chitiooligosaccharides into periplasmic space using chitoporin; and (3) catabolization. Chitin oligosaccharides in periplasmic space are degraded by ExoI into GlcNAc, which is taken up via the phosphotransferase system (PTS), whereas the $(GlcNAc)_2$ is taken up unchanged. $(GlcNAc)_2$ is implicated as true inducer of chitinolytic enzymes involved in catabolic pathway of marine bacteria.

responsible for this massive turnover and balancing marine ecosystems (Covazzi Harriague et al. 2008) (Figure 29.1). As described above, chitin is natural amino-polysaccharides produced annually in the marine biosphere alone with greater than 10^{11} tons, and is very important component of both the carbon and nitrogen cycles in marine environment at least. This large mass of highly insoluble polysaccharides is degraded so rapidly and is consumed by marine organisms including, via a multitude of tightly regulated genes encoding sort of enzymes such as chitoporin (Keyhani et al. 2000a), chitodextrinase (Keyhani and Roseman 1996b), *N*-acetylglucosaminidase (Chitlaru and Roseman 1996), deacetylase (Li et al. 2007), etc.

29.2.4 Core Enzymes in Chitin Catabolic Pathway

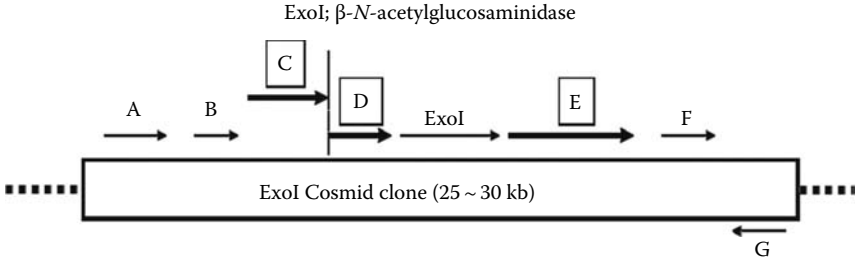
Chitin degradation and consumption in the marine bacteria are a complex process proceeding with several classes of enzymes involved in signal transduction systems. Despite chitin catabolic processing in the marine bacterium *V. furnissii* and *V. cholerae* is much more complex than being expected, only some genes and proteins in the chitin catabolic cascade have been identified. Excluding these bacterium, other specific genes and their transcriptional products validated previously in chitin catabolic pathway have not been identified up to date. In general, extracellular chitin sources derived from mostly outer shall debris of shrimps and crabs in ocean are hydrolyzed by extracellular chitinase(s), and the produced oligosaccharides can possibly be further utilized by many other organisms including *Vibrio* sp. *V. furnissii* and *V. cholera* are screened as chitinolytic marine bacterium that efficiently hydrolyze chitin into smaller molecules. Oligosaccharides produced by these bacteria are further diffused through chitoporin, which has been identified as a translocated membrane protein into the periplasmic space of bacteria. The combined action of two unique enzymes in the periplasm, a chitodextrinase and a specific β-*N*-acetylglucosaminidase (Bassler et al. 1991a; Chitlaru and Roseman 1996), yields two products, GlcNAc and $(GlcNAc)_2$. Particularly, the monosaccharide is taken up via the phosphotransferase system (PTS) (Meadow et al. 1987;

Chauvin et al. 1994; Bouma and Roseman 1996), whereas the disaccharide is taken up unchanged. The further catabolism of $(GlcNAc)_2$ gives *N*,*N*′-diacetylchitobiose, which can be catabolized by *Escherichia coli* and is transported/phosphorylated by the phosphoenolpyruvate:glycose phosphotransferase systems (Keyhani et al. 2000b), the major subject to address in-depth in the whole pathway of the chitin degrading mechanism in Vibrios.

According to the results of sequencing and comparing the homology on databank, there were three hypothetical genes out of eight genes in the chitin catabolic operon named as cosmid clone, which was isolated from genomic DNA of *V. furnissii* that has successfully been characterized (Figure 29.2). Each of the genes encoding hypothetical proteins that seemed to be involved in chitin catabolic pathway were cloned and overexpressed in *E. coli*. Proteins were purified, showed apparently homogeneous in SDS-PAGE, and subjected to characterize the enzymatic functions to determine the kinetic properties, respectively. Thus far, attempts to the molecular cloning of the periplasmic β-GlcNAcidase were not successful. Many attempts to clone the hypothesized cytoplasmic β-GlcNAcidase isolated a unique enzyme, a phosphorylase (Park et al. 2000). Although crude extracts of *V. furnissii* were shown to hydrolyze *p*-nitrophenyl-β-GlcNAc, the enzyme was assumed to be a typical bacterial chitobiase. Numerous unsuccessful attempts were made to clone this enzyme. Instead, an atypical periplasmic β-*N*-acetylglucosaminidase was identified, which has virtually no activity on $(GlcNAc)_2$ at the pH of sea water. The studies were based on the same approach, an attempt to clone the hypothetical chitobiase. Instead a novel enzyme was cloned and characterized successfully, a specific $(GlcNAc)_2$ phosphorylase that catalyzes the phosphorolysis of *N*,*N*′-diacetylchitobiose in the presence of ATP, resulting in the inversion of anomeric configuration ($\beta \rightarrow \alpha$) of $(GlcNAc)_2$ into GlcNAc and GlcNAc-α-1-P (Park et al. 2000).

An earlier study described that both enzymes encoded by periplasmic *b*-*N*-acetylhexosaminidase gene (*exo*I) and an *N*,*N*′-diacetylchitobiose phosphorylase gene *chb*P from *V. furnissii* are critical in the chitin degradation cascade in Vibrios. Furthermore, sequence analysis of the cloned *exo*I from *V. furnissii* suggested that there were two putative open reading frames (0.8 and 1.7 kb) located upstream of the gene named as a specific $(GlcNAc)_2$ phosphorylase *chb*P. Subsequent studies on the two open reading frames, designated *gsp*K (0.8 kb) and *bgl*A (1.7 kb), were conducted with other bacterial strain *V. cholerae*, respectively.

Several methods have been used for measuring the rate of sugar-P synthesized by specific kinases and ATP, or, by the phosphoenolpyruvate:glycose phosphotransferase system (PTS). Technically, the quantity of ADP produced by the kinase from ATP used as substrate was determined with P-enolpyruvate, pyruvate kinase, NADH, and lactate dehydrogenase. GlcN was omitted from



ORFs identified as having Homology to;
A: Oligopeptide permease; B: Oligopeptide transport ATP binding protein
or Oligopeptide permease; C: Hypothetical protien; D: Hypothetical protein;
E: Hypothetical protein; F: Phosphomannomutase; G: ABC transporter.

FIGURE 29.2 ExoI cosmid clone. Genes involved in chitin degradation pathway are cloned and sequenced. According to the result of sequencing and comparing the homology on databank, each of them was further characterized, including three hypothetical genes in the chitin catabolic operon named as an ExoI cosmid clone, which was isolated from genomic DNA of *Vibrio furnissii*.

controls to correct for any ADP formed from contaminating enzymes, such as ATPase. Upon optimizing the reaction condition for ATP-dependent specific kinase, the substrate specificity of the enzyme, GspK was investigated. Following sugars such as galactosamine, mannosamine, glucose, GlcNAc, $(GlcNAc)_n$, n = 2–3, $(GlcN)_2$, lactose, sucrose maltose, 2-deoxyglucose, galactose, and mannose were used to determine the substrate specificity of ATP-dependent specific kinase. Based on the results, authors demonstrated that a novel enzyme named as GspK has shown the high specificity against glucosamine (Park et al. 2002b).

In addition, in order to complete the chitin catabolic pathway in Vibrios, a gene that followed *gsp*K in chitin catabolic operon was molecularly cloned into *E. coli*. The gene, which encoding a protein BglA, was overexpressed and purified to be homogeneous protein. Kinetic parameters of enzyme against both an artificial substrate *p*NP-glucoside and natural substrate cellobiose $(Glc)_2$ were calculated. Interestingly, BglA displayed no activity toward any dimer sugar substrates tested including $(GlcNAc)_2$, chitobiose $(GlcN)_2$, chitin oligosaccharides $(GlcNAc)_n$ (n = 3–5), trehalose, lactose, maltose, and sucrose. More importantly, it exhibited no activity with positional isomers of cellobiose such as sophorose, laminaribiose, and gentiobiose. Furthermore, no activity was detected with *p*NP-β-GalNAc, *p*NP-β-glucose-6-phosphate, or with *p*NP-$(GlcNAc)_n$ (n = 1–3). However, BglA was active with the higher β-1,4-Glc-linked oligosaccharides; cellotriose, cellotetraose, and cellopentaose. Due to the enzyme specificity to the substrates, BglA has been identified as a unique *exo*-type β-glucosidase (Park et al. 2002a).

29.2.5 Complexity of Chitin Catabolic Pathway in Vibrios

Many different types of genes and proteins are involved in the completion of chitin catabolic pathway at least in Vibrios, as described above. Although less and limited information are provided from other organisms, it may seem to be similar with Vibrios. Briefly, chitin oligosaccharides, $(GlcNAc)_n$, produced by combinational function of extracellular and/or intracellular chitinase(s) can enter into the periplasmic space via a specific porin designated as a specific membrane protein. Further study should address the size of oligosaccharides that can enter the periplasmic space without changing of length. However, smaller sizes of monosaccharides and $(GlcNAc)_2$ penetrated the cell envelope through constitutive porins also. It might be dependent on the species of organisms. In the periplasmic space, $(GlcNAc)_n$ oligomers are further converted to two products GlcNAc and $(GlcNAc)_2$ by two unique enzymes, a chitodextrinase and a β-*N*-acetylglucosaminidase (Bassler et al. 1991b; Keyhani and Roseman 1996a,b). GlcNAc and $(GlcNAc)_2$ are then rapidly consumed and metabolized in intact cells. Earlier study demonstrated that there are two other possible sources for the key intermediate compound, GlcNAc-6-P. One is known to be phosphoenolpyruvate:glycose phosphotransferase system, a key system for the monosaccharide, GlcNAc, which is taken up by the specifically by Enzyme II^{Nag} (Bassler et al. 1991b). The gene involved in phosphotransferase system for GlcNAc has been cloned from *V. furnissii*, and the protein has been characterized. The overall reaction of the transport process of GlcNAc is: P-enolpyruvate(in) + GlcNAc(out) → GlcNAc-6-P(in) + pyruvate(in). The further metabolism of GlcNAc-6-P involves two steps, deacetylation and deamination, yielding fructose-6-P, NH_3, and acetate. In *V. furnissii*, $(GlcNAc)_2$ generated both outside the cell by chitinases and in the periplasm from higher oligosaccharides is taken up unchanged by a specific transporter. Another one can be explained by the function of *N*,*N*′-diacetylchitobiose phosphorylase, cleaves disaccharide yielding GlcNAc-1-P and GlcNAc (Park et al. 2000). Consequently, the GlcNAc-1-P is converted to the 6-P by a specific mutase. In addition, the third source of GlcNAc-6-P is a GlcNAc-specific ATP-dependent kinase that is found in *V. furnissii*. Subsequently, the free GlcNAc generated from the disaccharide by the phosphorylase or entered from outer space possibly unchanged is converted to GlcNAc-6-P by this kinase. Now it is acceptable as a routine pathway for chitin-degrading mechanisms in marine bacteria, in particular *V. furnissii* or *V. cholera* (Byung-Ok Jung and Park 2008).

29.3 CONCLUDING REMARKS

To date, chitin degradation in the marine biosphere comprises at least four major steps as shown in Figure 29.1: (1) bacterial sensing of chitin either by random collision or chemotaxis; (2) attachment to the chitin resource to utilize as carbon and/or nitrogen source; (3) expression of a multitude of extracellular chitinolytic enzymes and other related proteins involved in the catabolism of chitin and its oligosaccharides; (4) uptake and catabolize the hydrolysis products in intact cells. Despite the discovery of many genes and proteins involved in the processing of chitin catabolic cascade in Vibrios, original sources of GlcN and cellulooligosaccharides are yet to be identified. One of possibilities is that the free GlcN might be obtained from the function of GlcNAc deacetylase in either cytoplasmic or periplasmic space. As described above, then the free GlcN is converted to GlcN-6-P by glucosamine-specific kinase, GspK, and/or ATP dependent kinase (Park et al. 2002b). On the other hand, cellulooligosaccharides $(Glc)_n$ derived from source of unknown origin are converted to glucose by exo-β-glucosidase, and utilized to the carbon source in Vibrios (Park et al. 2002a). It is not clear yet where $(Glc)_n$ comes from, consequently, the physiological role of both BglA and GspK raises important questions for further deep understanding of the chitin catabolic pathway in Vibrios and presumably also other chitinolytic organisms (Figure 29.3).

Whereas, less attention on chitosan degradation pathway in chitosanolytic organisms was paid, despite several genes encoding chitosanolytic and relative enzymes were cloned and sequenced. Interestingly, chitosan also could be hydrolyzed to be chitooligomers by combinational functions of nonspecific enzymes including cellulases, proteases, lipases, and pepsins. This may occur from the structural similarity of chitin, chitosan, and/or cellulose, which are all linear polymers of D-glucose linked by β-1,4-glycosidic linkages. It seems to be that those enzymes could not recognize precisely the group of C2 position in *N*-acetylglucosamine, glucosamine, and glucose residues when

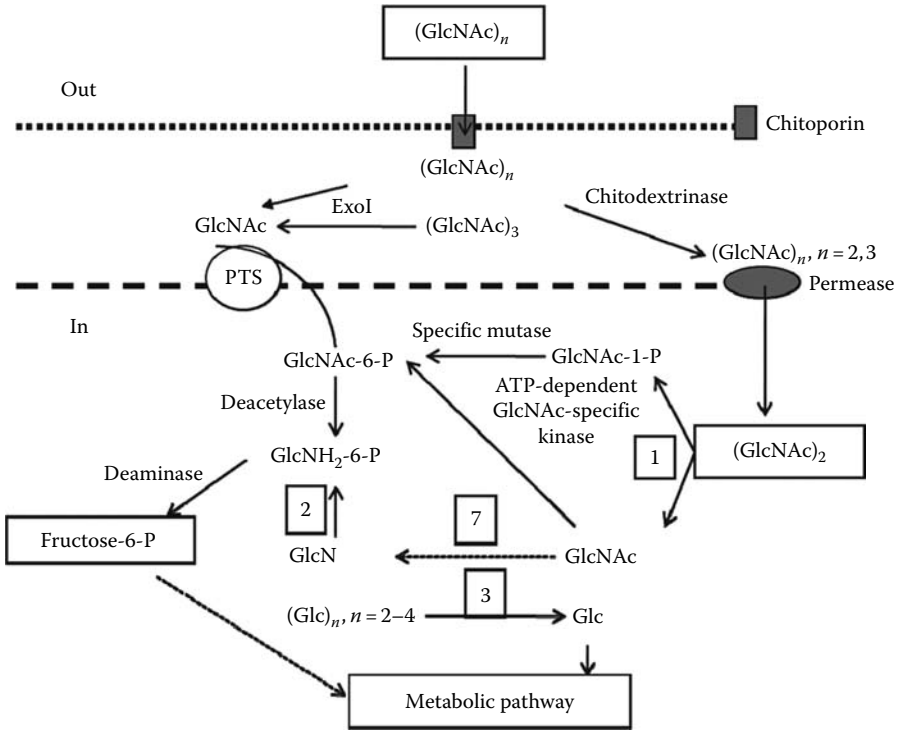


FIGURE 29.3 Sophisticated catabolic mechanisms of chitooligosaccharides in vibrios. In earlier studies, many enzymes involved in the signaling pathway were cloned and characterized. 1, *N*,*N*′-diacetylchitobiose phosphorylase (ChbP); 2, glucosamine-specific kinase (GspK); 3, β-glucosidase (BglA).

enzyme–substrate complex was formed. Nevertheless, chitooligosaccharides are known to be biologically active materials, especially to the activity of antitumor due to its cationic properties exerted by amino groups of chitooligosaccharides. In addition, their molecular weight that is extremely important to play a major role in biological activities such as antifungal activity, immune-enhancing effects, antimicrobial activity, and antioxidant activity. Probably those activities attracted more attention of many research scientists to the basic studies related to the biological role of chitooligosaccharides in clinical research rather than perform deeper investigation of catabolic pathway of chitosan and its derivatives in any specific organisms.

In conclusion, although many genes and their protein products with potential functions in chitin catabolic pathway including three unique genes are now cloned, much more extensive investigations need to be conducted to map out the whole cascade of the chitin degradation pathway in the marine bacterium Vibrios, including chitosan degradation pathway for other chitinolytic organisms.

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30 Medical Applications of Chitin and Chitosan: Going Forward

Eugene Khor

CONTENTS

30.1 INTRODUCTION

From the 1950s onward, research of chitin and chitosan with regard to their production, chemistry, biology, and applications has been intense. One of the key areas of promise for chitin and chitosan is the area of biomedical applications. A comprehensive survey of this aspect during the period 1950–2000 was covered in a 2001 review (Khor 2001). Since the turn of the twenty-first century, there has been no let up in the research of chitin and chitosan for biomedical applications, and publications continue to appear in the scientific literature. One interesting observation is that publications from new research groups from previously non-participative centers are appearing and increasing. Conversely, publications from established or traditional authors have seen a decline in their output and some are no longer contributing. The author notes the passing of Professor S. Hirano, the retirement of many of the twentieth-century pillars of this research community, and the elevation of others to senior administrative positions. It is therefore fitting that a new generation of scientists is filling the void. This portends well for the future of chitin and chitosan research.

In the author's review nearly 10 years ago, the processes that should take place for progress from research into realization of biomedical products were introduced. The premise for the best way of perpetuating chitin and chitosan biomedical research interests that attracted recurrent R&D funding was the realization of chitin- and chitosan-based biomedical products from ongoing research activities. This completes the research cycle between scientific research funding and commercialization (Figure 30.1).

In the ensuing years, the landscape pertaining to the biomedical applications of chitin and chitosan has changed. The most significant being the emergence of companies introducing primarily chitosan-derived biomedical products. It is encouraging to note that two of the companies featured

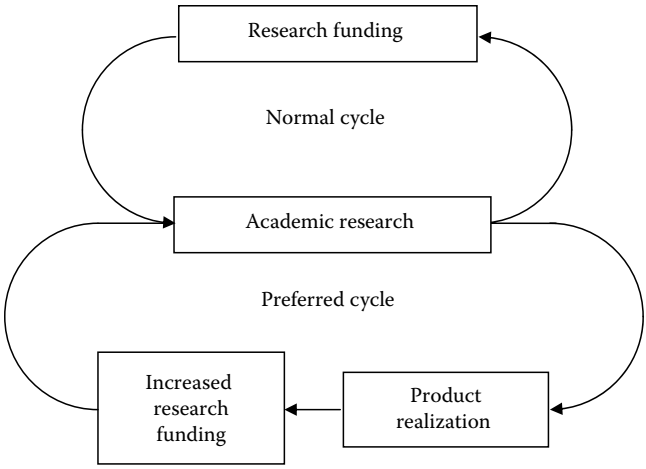

FIGURE 30.1 Expected impact of product realization on research funding.

below are the outcomes of active steps taken to move chitin and chitosan research from the academic lab bench to practical medical products that are vital for continued research. This has been augmented by a select group of publications in the scientific literature that report on studies specific to these chitosan-based biomedical products. This is in contrast to most articles that continue to focus on their potential biomedical applications.

Will there come a time when publications among potential and real products are more balanced in number? Will chitin and chitosan-containing biomedical products proliferate? It is the intention of this chapter to review the present state of this implicit academia–industry relationship and indicate some of the likely areas that will see increased activity going forward in this exciting time of chitin and chitosan research for biomedical applications.

30.2 DEMONSTRATED BIOMEDICAL PRODUCTS

A relevant starting point is to summarize the commercial successes of chitin and chitosan as they pertain to medical applications. Chitin and chitosan-based medical products have been in the market for some time, especially in Asian countries. These medical products are typically for local consumption having passed through the stringent regulatory requirements of individual countries where the products are sold.

For a wider acceptance, conformance to internationally accepted practices and standards is desired. These requirements are rigorous, entailing chitin and chitosan materials and their products to have been produced under some form of Good Manufacturing Practice (GMP) process and evaluated under Good Laboratory Practice (GLP) procedures. Featured next, by no means exhaustive, are four of the better known examples.* Collectively, they demonstrate that chitin and chitosan biomedical products conforming to the present international regulatory requirements can be attained.

Bio Syntech Canada Inc. is a Laval, Quebec, Canada, company founded on technology derived from a Montreal-based institution (Bio Syntech Web site 2009). Its platform appears to be chitosan-containing thermogels applied in regenerative medicine for cartilage repair, chronic wound healing, heel pain relief, bone filling, and invertebral disc restoration (Hoemann et al. 2005). Biosyntech also produces ultrasan™, an ultrapure chitosan. According to its Web site, the company produces its products under ISO 9001:2000 certification, have 73 patents granted and 40 pending with several applications in "various stages of development."

* It is stated categorically that the author has no association with any of the companies featured.

HemCon Medical Technologies Inc. is a Portland, Oregon based company (Hemcon Inc. Web site 2009). Its first product was Hemcon®, a chitosan hemostat wound dressing that was first introduced in 2003. Other chitosan-containing medical products that are elaborations of its core wound dressing technology and expertise have since been introduced. One interesting new feature appears to be nanoSpider™ that permits nanofibers, presumably of chitosan, to be produced. This opens up a myriad of possibilities for future utilization.

Marine Polymers Technologies Inc. is a Danvers, Massachusetts based company. No official Web site for this company has been found. The core technology is chitin or 100% poly-*N*-acetylglucosamine and is another academic institution developed technology. Listed among its products are liquid bandage, hydrophilic wound dressing, wound dressing, and adhesive bandage (medical device internet portal search). The evaluation of one of its product, the rapid deployment hemostat (RDH) bandage, has been reported (Jewelewicz et al. 2003).

NovaMatrix is a business unit of FMC Healthcare Ventures and associated with FMC Biopolymers, a subsidiary of FMC Corporation that produces chitosan products according to cGMP, ISO9001:2000 and ISO13485:2003 standards (NovaMatrix Web site 2009). Some of their products are listed for trial only and not ready for human use.

This short summary demonstrates that each company has selected a niche application to focus on for their success. The wound dressings have been in the market for some time, while the thermogel applications are apparently still in the regulatory approval process. This is more an indication of the divide in meeting regulatory requirements between external medical products and internal implants rather than product shortcomings. Clearly, this overview indicates that chitin and chitosan-based medical products can be termed commercially successful and this augments well for the future of chitin and chitosan in biomedical research and applications that reach commercialization.

Two facets require further elaboration. First, the perennial "bone to pick" inhomogeneity issue of this biopolymer. Bio Syntech, HemCon, and NovaMatrix offer good high purity grade chitosan products for sale, opening opportunities for utilizing standard materials in biomedical applications research. This contrast to the existing situation where most scientific work reported utilize what can be termed industrial grade chitosan, obtained from in-house preparation methods or from chemicals-type manufacturers and suppliers. Consequently, the molecular weights and degrees of acetylation or deacetylation of the chitins and chitosans reported in journal articles are generalities at best, and within the same article, variation may exist even from sample to sample. Clearly, scientific reporting would benefit from using some form of standardized materials if the heterogeneous nature of this biopolymer is to be addressed adequately for future ease of reference. Whether this can be achieved at reasonable costs with the high purity grade material is the real issue.

Second, the medical products advanced by these companies make use of one base material, chitosan or chitin. This implies that there is still a lot of scope for developing chitin and chitosan biomedical products. Some possibilities are

1. Investigating other clinical conditions where the base materials could be applied
2. Extending the base materials' usefulness by focusing on their hydrogel and fiber forms as well as films and nano configurations
3. Developing defined lower molecular weights or oligomeric forms of chitin and chitosan as future application potential
4. Continue to explore combination materials of chitin and chitosan with other polymeric or structural materials
5. Developing chitin and chitosan derivatives and characterizing them; their known properties could be matched against potential biomedical requirements

The outlook is therefore extensive. Discussed below are the author's views of some of the key areas where development will or has to take place in the next 10 year horizon.

30.3 DIRECTIONS FOR THE FUTURE

While forecasting or predicting the future may not be a function of science, the issues that confront chitin and chitosan research in the biomedical applications area in the near term are self-evident. Three aspects summarized in Figure 30.2 are put forward as desirable to occur.

30.3.1 Production

First, there remains a continuing need for standardized chitin and chitosan materials to be readily available for purchase at reasonable costs. This is advocated as one channel that can eliminate the persistent ambiguity of the purity, degree of acetylation/deacetylation, molecular weight, and other standard properties found in the scientific literature for this biopolymer. The proliferation of chitin and chitosan in biomedical research and eventually biomedical products demands that this take place.

While the materials from the companies mentioned above can be a resource, there is still scope for new suppliers to provide chitin and chitosan materials produced in a standard and controlled manner that is reliable and are properly characterized to emerge. This is because an ultrapure grade for biomedical applications can cost upward of US$50,000/kg (Dornish and Kaplan 2003). When a pure grade with the appropriate documentation that is affordable is made available for purchase by researchers, direct comparison of research results that are unambiguous in the scientific literature would be more easily facilitated (Figure 30.3). Of course, this does not exclude variations in individual experimental designs and practice but the most problematic issue of starting material integrity is resolved. Once confirmation of favorable experimental results is realized, utilizing the high-end materials is easier to justify and is also usually at the stage where academic research transforms into product development.

This awareness has been increasing for some time. Dornish et al. announced in 2001 some of the work that was carried out by an ASTM committee on standardizing chitosan for tissue-engineered medical products that were reiterated at the 9th International Chitin Chitosan Conference (Dornish et al. 2001, Dornish and Kaplan 2003). It is likely that this "missing link" in the research–applications interaction will be closed in the not too distant future.

The other area of development that would benefit researchers is the availability of several molecular weights of chitin and chitosan produced under the same stringent production criteria.

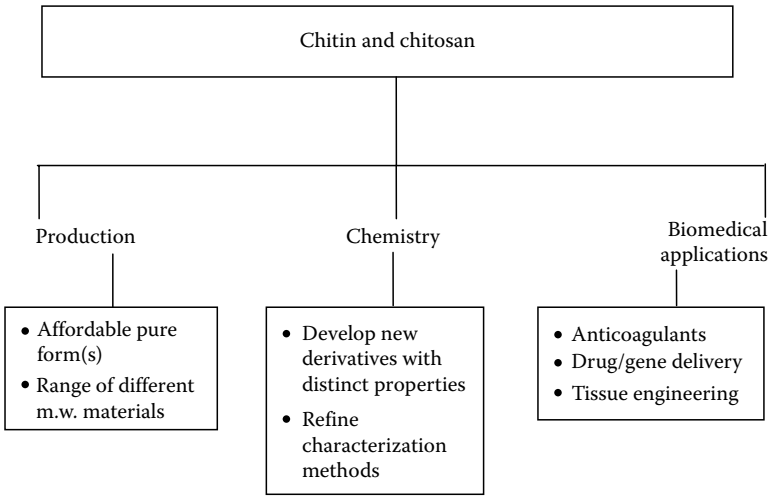

FIGURE 30.2 Anticipated directions for chitin and chitosan biomedical research and applications.

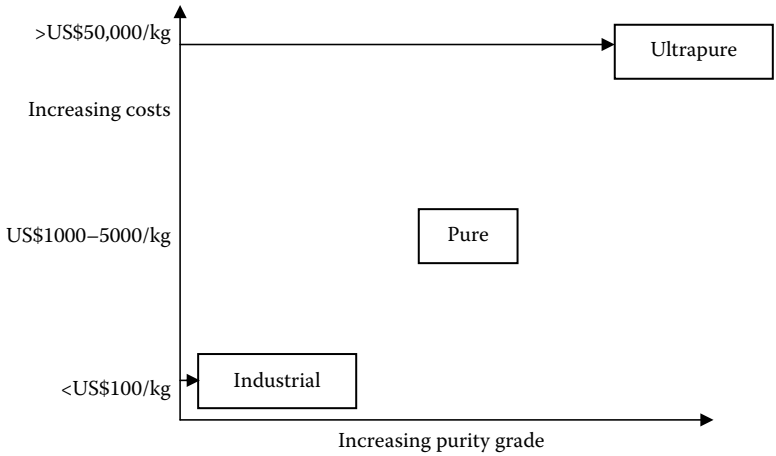

FIGURE 30.3 Costs–purity relationship of chitin and chitosan. (Adapted from Dornish, M. and Kaplan, D., *Adv. Chitin Sci.*, VII, 152, 2003.)

The 500, 250, 100, and 50 kDa molecular weights are proposed as suitable sizes to provide a range flexible enough for various investigations. There is also a possibility of extending this to oligomers, but that would depend on demand that suppliers in due course can meet.

These two key production concerns of chitin and chitosan when managed properly can be the springboard for chitin and chitosan research to be set on a level playing field. This can only energize research interactions globally and establish chitin and chitosan as one biopolymer isolated solely from biological sources that can match synthetic polymers as biomaterials with defined purity characteristics.

30.3.2 Chemistry

One of the most exciting features of chitin and chitosan are their potential for chemical derivatization. Through the years, it has been demonstrated with various reaction conditions and reagents that the C6-OH, C3-OH, and N2-H sites can produce derivatives that not only provide variety but new channels suited for biomedical applications.

30.3.2.1 Chemical Derivatization

As stated above, the present biomedical products utilize only the base materials. Chemical derivatization is the way forward to realize the full potential of chitin and chitosan (Alves and Mano 2008). The well-known C6-carboxymethyl-chitin and chitosan would be an ideal starting place (Raimunda and Campana-Filho 2008). However, this work again started with the alkali–chitin process that is known to be inefficient and associated with inhomogeneity concerns. Another recent work is the preparation of *N*-hydroxyacryl-chitosan that utilizes chitosan from a commercial source without further treatment (Maa et al. 2008). Phosphorylated chitins and chitosans are another example of chemical derivatization that yields soluble compounds (Jayakumar et al. 2008).

The advantage of chemical derivatives has always been their solubility potential. This ability alone lends the biomacromolecule to more thorough chemical characterization, for example, molecular weight determination by GPC, poorly achieved with chitin and challenging with chitosan. The use of lower molecular weight materials would alleviate the situation somewhat, but truly soluble systems especially in water would transform the evaluation of these biopolymers that is vital for biomedical applications. Coupled with the potential for new desirable properties a chemical derivative can provide, this aspect will increasingly attract attention.

A further variation is that of generating new forms from these derivatives or the chemical derivatization of a pre-determined form such as fiber or hydrogel of chitin or chitosan. For example, Hirano et al. demonstrated the production of *N*-acylated-chitosan fibers by wet spinning of their solutions (Hirano and Midorikawa 1998). This process was extended to chemically derivatizing chitosan when in the fiber form (Hirano et al. 2000). Wan et al. have also shown that chemical derivatization of chitin can take place after forming a hydrogel or film (Khor et al. 1997, Wan et al. 1997). Therefore, it is likely that research in developing and characterizing new chemical derivatives will flourish.

30.3.2.2 Characterization

There is no doubt that the characterization of chitin and chitosan materials has been the subject of much work and debate. Molecular weight and the degree of acetylation (D.A.) or deacetylation (D.D.) remain the primary properties of interest. Residual protein, other impurities and ash content are some of the other information sought. The use of GPC and intrinsic viscosity measurements for molecular weights persist, while NMR, IR, UV, and elemental analysis have all been used to report on the D.A. and D.D. (Kasaai 2009). It is now clear that where possible, NMR is the method of choice to evaluate D.A. and D.D., while IR, UV, and elemental analysis are still used where the sample mode does not permit easy access for NMR elucidation.

It is anticipated that a consensus platform to determine these issues at various chitin and chitosan conferences together with industry input will resolve this aspect with time.

30.3.3 Biomedical Applications

Finally, a discussion of the likely events to take place in the biomedical applications of chitin and chitosan is germane to this chapter. There is no end to the imagination of researchers in originating new biomaterials. Many reports detail the combination of chitosan with other synthetic polymers, different forms and other new iterations that contain the biopolymer. The only requirement that should be followed is to utilize standard materials when they become available and affordable. Traditionally, several areas have been championed and warrant a status review.

30.3.3.1 Rethinking Anticoagulant and Drug/Gene Delivery Applications Studies

For innumerable years, the interest in sulfated chitins and chitosans have centered on their potential as anticoagulants that can substitute for heparin (Jayakumar et al. 2007). Many studies have shown that their anticoagulant properties are superior to heparin (Zou and Khor 2009). Despite the promise, should research of sulfated chitin and chitosan as potential anticoagulants continue? To answer this question, it is imperative to know how anticoagulation research and applications have progressed, as anticoagulation therapy utilizing heparin has itself undergone a progressive evolution. Heparin is a complex high molecular weight substance extracted from animal sources and was used for thrombosis treatment for several decades (Middeldorp 2008). Subsequently, the low molecular weight heparin produced by enzymolysis of high molecular weight heparin was introduced as a more effective replacement. Today, the synthetically made pentasaccharide, fondaparinux promises to be the ultimate replacement in the evolution of this drug.

It is therefore timely to decide whether further research along this direction for sulfated chitin and chitosan should continue. Certainly if the aim was to develop a replacement anticoagulant to heparin, the performance may not match that found in the third generation derivative, the pentasaccharide version of heparin. In addition, no work to date has been reported regarding the antidote to reverse the anticoagulation effects of sulfated chitins and chitosans, a necessary feature if anticoagulation were to be effective. Therefore, it may be realistic to re-evaluate interest in this aspect of research. This does not preclude investigating applications where sulfated chitin and chitosan may still have an advantage such as in anticoagulant coatings or fibers that can be spun into a useful conduit. It would also be useful to investigate other applications where the sulfate moiety is required, found useful or desired.

The applications of chitin and chitosan as drug and gene delivery carriers also continue to be popular. In a recent review, Lai and Lin summarized the strategies that are being pursued for chitosan in nucleic acid delivery (Lai and Lin 2009). Piquette-Miller et al. report work on preparing chitosan–phospholipid as minimally invasive systems for drug delivery as well as tissue engineering (De Souza et al. 2009). Here again, it is useful to pause and ascertain the state of progress in the development of competing carriers.

Parallel to these chitosan-based systems, synthetic chemists have been developing drug delivery polymers as potential carriers as well. One well-known system is the dendrimers (Boas and Heegaard 2004). Sophisticated polymers can be obtained by a step-by-step growth for several generations of this "star" polymer system. The chemical synthesis is improving and drug loading studies no doubt follows.

The purpose of structuring this section with a cautionary tone was to impress the need to constantly evaluate the competition in developing biomedical applications. As stated earlier, generating biomedical products from research is desirable. One consideration is obtaining regulatory approval for medical products that is at best a complex process. Take the instance of the synthetically produced pentasaccharide compared to sulfated chitin and chitosan. The biopolymer would be compared to a material that has clearly defined production profiles and would be expected to undergo a more straightforward regulatory approval process compared to the biopolymer. Additionally, the role of an anticoagulant and drug delivery carrier is primarily a chemistry-type function where the synthetic molecule again is better placed. Knowledge of what abounds in other associated research areas is important for chitin and chitosan researchers. Therefore, while not advocating the complete abandonment of investigating chitin and chitosan for anticoagulant and drug/gene delivery applications, it is suggested that there is a need to attenuate unreasonable expectations. In an increasing competitive research environment, defining their system's advantage over the synthetic analogs is the rationale that would support justification for further research to maximize finite resources.

30.3.3.2 Tissue Engineering and Wound Healing

Tissue engineering applications utilizing chitosan to perform the scaffold function have been popular since this field took off in the early 1990s. Due to its solubility properties, this has been largely confined to lyophilization of chitosan solutions, porogen systems, films, and fibers. Some of the ways that chitosan can be utilized for tissue engineering, drug delivery, and wound healing have been reviewed (Nicodemus and Bryant 2008, Priya et al. 2008, Tuzlakoglu and Reis 2009).

The use of chitin materials for wound healing has also been extensive. A recent review summarizes how much is now known about the interaction of chitin materials with the wound bed in promoting healing (Muzzarelli 2009). The hemostatic properties of chitosan are again highlighted, while the role played by chitin materials interacting with various factors and enzymes are elaborated. A discussion of various forms of chitin and chitosan containing gels and films and their role in cartilage repair, nerve regeneration, and other uses were also included.

One area where focus of much work can be addressed is that of generating new forms especially hydrogels, exemplified by work done by Nie et al. (Li et al. 2009). The use of nanotechnology is also an exciting prospect in generating new shapes (Fernandez et al. 2009). Finally, possibly the "Holy Grail" direction would be to develop a melt processible form of the biopolymer. Were this achievable, it would open an extensive processing advantage in utilizing this biopolymer.

30.4 CONCLUSION

The biomedical applications promise of chitin and chitosan has always been there and inroads have certainly been made since the late 1990s. Medical grade materials are now more available and chitosan-based biomedical products have been introduced. This is a good indicator of future successes.

The prospect for good purity grade materials and a choice of molecular weight ranges at an affordable price to advance the scientific research process appears promising. The directions for future research remain multi-faceted. This would probably encompass chemical derivatization and combination materials. The types of biomedical applications research need to be refined to take into account the advantages of the biopolymer system for maximum impact. Product development must follow and should be encouraged.

ACKNOWLEDGMENT

The author would like to thank the National University of Singapore for financial support of his Biomaterials and Chitin & Chitosan Research programs for the past 20 years.

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31 Radiation Functionalization and Applications of Chitosan and Its Derivatives

Maolin Zhai, Long Zhao, Ling Huang, Ling Xu, Liyong Yuan, and Min Wang

CONTENTS

31.1 INTRODUCTION

Chitosan, a copolymer of glucosamine and *N*-acetylglucosamine units linked by one to four glucosidic bonds, is commercially obtained from chitin, which is one of the most abundant natural amino polysaccharide. Chitosan could be converted by using proper reagents into a number of *O*-alkyl and *O*-acyl derivatives. Chitosan also behaves as a moderately basic cationic polyelectrolyte, which readily forms salts with acids. In addition, the presence of the primary amino group in chitosan offers further possibilities for modifications such as *N*-acylation, *N*-alkylation, and *N*-alkylidenation. Therefore, numerous varieties of chitosan derivatives can be synthesized from this nature's abundant polymer.

Over the last few years, there has been a rapidly expanding interest in chitosan and its derivatives, both from a fundamental viewpoint "with regards to their unique biological and chemical reaction mechanism" and an application viewpoint "in terms of their great biotechnological and industrial usefulness." Chitosan and its derivatives have been reported to have a variety of applications in industrial and biomedical fields, due to their nontoxicity, biodegradability, biocompatibility, and antibacterial activity. Among them, carboxymethyl chitin (CM-chitin), carboxymethyl chitosan (CM-chitosan), and dihydroxypropyl chitosan (DHP-chitosan) are highly hydrophilic polymers, being widely used because of their good processability and water solubility, and thus providing the possibility for green fabrication approach. They have been successfully used in the food industry, water treatment, textiles and paper, detergents, cosmetics, pharmaceuticals, and biomedical areas. These applications are attracting a lot of research interests to develop functional polymers from chitosan derivatives.

Radiation processing is a very convenient tool for the modification of polymer materials. Under γ-rays or electron beams irradiation, free radicals are stimulated from polymer matrix and circumstance media, followed by a serial of free radical recombination or free radical quenching, which is essential for the proceeding of radiation cross-linking, degradation, and grafting. Due to the abundance of polymer matrix and modification routine, plenty of functional materials have been and could be developed by radiation processing for various applications.

Recently, radiation effects, i.e., radiation degradation and radiation cross-linking, on chitosan and its derivatives have been investigated to develop their application in the fields of health, environment, and agriculture. It has been well known that chitosan and its derivatives can be degraded by the scission of glycoside bonds under irradiation. These radiation-degraded products have lower molecular weight and enhanced antibacterial ability, leading to their marvelous prospects in the applications such as plant-growth promoter, phytoalexin, and so on.

On the other hand, the radiation cross-linking of chitosan derivatives has resulted in the production of biodegradable hydrogels. For example, CM-chitin and CM-chitosan were found to be cross-linked at high-concentrated aqueous solution (more than 10%, paste-like state). These hydrogels are excellent water absorbents with satisfying biodegradability and biocompatibility, which could be widely used in various fields such as biomedical, agriculture, and cosmetic. Some recent progress concerned with the radiation modification of chitosan and its derivatives will be introduced in this chapter.

31.2 RADIATION-INDUCED DEGRADATIONS AND THEIR APPLICATIONS

In many applications, molecular weight is a very important parameter to be controlled. Recently, radiation technique has been used widely to produce low-molecular-weight chitosan by degradation. Several properties of chitosan and its derivatives are correlated to their molecular weight. It has been well known that chitosan and its derivatives can be degraded due to the scission of glycoside bonds by irradiation. A random cleavage of glycoside bonds in the main chains of polysaccharide, initialized by the radicals formed on the chains, is the predominant reaction during

polysaccharide degradation. Degradation behavior was dependent upon irradiation conditions, for example, the required dose to prepare low-molecular-weight sample was lower in aqueous solution than that in solid state. This may be ascribed to the contribution of •OH radicals formed by radiolysis of water. Pulse radiolysis and laser photolysis have been used to elucidate the radiolysis mechanism of chitosan and its derivatives. Compared to the traditional methods such as acidic hydrolysis or enzymatic treatment, radiation method prevents the usage of initiators, resulting in high-purity products. Therefore, radiation method is simpler as well as more environmentally friendly compared to conventional ones. This radiation-degraded low-M_w chitosan and its derivatives are found to induce antioxidant and biological activities such as antimicrobial activity, promotion of plant growth, suppression of heavy metal stress, and phytoalexins induction. What is more, the radiation products of chitosan can be used to synthesize natural oligosaccharides-derived ionic liquids at room temperature. Thus in the following section, the degradation behavior of chitosan, CM-chitosan, and some typical applications will be described.

31.2.1 Radiation-Induced Degradation of Chitosan

31.2.1.1 Degradation in Solid State

Chitosan in the solid state could be degraded by γ-irradiation, and usually a 100 kGy dose is applied to obtain low-molecular-weight chitosan (Li et al. 2001). The mechanism may be ascribed to the direct action of radiation on the chitosan chain (reactions (31.1) and (31.2)):

$$RH \xrightarrow{\gamma\text{-ray}} R^{\bullet} + {}^{\bullet}H \tag{31.1}$$

$$R^{\bullet} \longrightarrow R_1^{\bullet} + R_2 \tag{31.2}$$

31.2.1.2 Degradation in Diluted Aqueous Solution

Water radiolysis is the main effect of the γ-irradiation of diluted aqueous solutions. In the case of diluted aqueous solutions of polysaccharides, •OH and H atoms formed from water radiolysis are able to abstract hydrogen atoms from the polymer to form the macroradicals (reactions (31.3) through (31.5)). The subsequent reactions of macroradicals can be chain scission (reaction (31.6)), hydrogen transfer, inter- and intramolecular recombination, and disproportionation of macroradicals. Chain scission leads to a decreasing in the molecular weight of the polymer.

$$H_2O \xrightarrow{\gamma\text{-ray}} H_2, H_2O_2, e_{aq}^{-}, {}^{\bullet}H, {}^{\bullet}OH, H_3O^{+} \tag{31.3}$$

$$PH + {}^{\bullet}OH \longrightarrow P^{\bullet} + H_2O \tag{31.4}$$

$$PH + {}^{\bullet}H \longrightarrow P^{\bullet} + H_2 \tag{31.5}$$

$$P^{\bullet} \longrightarrow P_1^{\bullet} + P_2 \tag{31.6}$$

The degradation rate of chitosan in aqueous solutions increases with decreasing polymer concentration (Figure 31.1). It is mainly caused by the enhanced •OH mobility rising with the reduced viscosity of diluted solution (Wasikiewicz et al. 2005b). Moreover, in diluted solutions the distance between

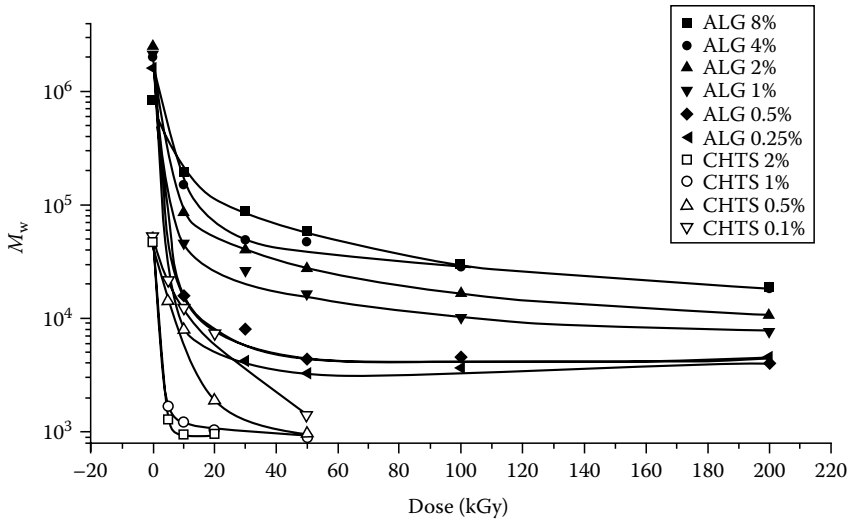


FIGURE 31.1 γ-irradiation degradation of sodium alginate and chitosan in aqueous solutions of different concentrations, irradiated at different doses. (From Wasikiewicz, J.M. et al., *Radiat. Phys. Chem*., 73, 287, 2005b. With permission.)

two radicals located on neighboring polymer chains becomes larger. It significantly decreases their recombination possibility, which reduces the possibility of cross-linking and thus favors degradation. The most pronounced decrease in molecular weight occurs at doses lower than 50 kGy, and after that molecular weight is close to the oligomer level.

The molecular weight of chitosan can be conveniently measured by the viscosity method. The viscosity of chitosan is measured at 25°C ± 0.1°C in 0.1 mol/L CH_3COOH/0.2 mol/L NaCl buffer solution with a capillary viscometer. The intrinsic viscosity [η] is calculated according to an empirical equation (31.7) (Fan et al. 2002):

$$[\eta] = \frac{(\eta_{sp} + 3\ln \eta_r)}{4C} \tag{31.7}$$

where

η_{sp} is the specific viscosity
η_r is the relative viscosity
C is the concentration of chitosan solution (g/mL)

The M_η is calculated using the Mark–Houwink equation (31.8):

$$[\eta] = KM_\eta^\alpha \tag{31.8}$$

where

$K = 1.81 \times 10^{-3}$
$\alpha = 0.93$

Degradation process can be described in terms of the changes of molecular weight due to the scission of polymer chain and its efficiency can be estimated by a radiation yield of scission G_s (mol/J) according to Equation 31.9 (Janik et al. 2003):

$$G_s = \frac{2c}{Dd}\left(\frac{1}{M_w} - \frac{1}{M_{w0}}\right) \tag{31.9}$$

where

c is the concentration of polymer in solution (g/dm^3)
D is the absorbed dose (Gy)
d is the solution density (kg/dm^3)
M_{w0} and M_w are the weight-average molecular weight of polymer before and after irradiation, respectively

The reduction in molecular weight of polymer could be described by Equation 31.10 (Tsaih and Chen 2003):

$$\frac{1}{M_t} = \frac{1}{M_0} + \frac{kt}{m} \tag{31.10}$$

where

k (h^{-1}) represents the rate constant of radiation degradation
m is the molecular weight of polymer monomer unit
M_t and M_0 are the weight-average molecular weights of chitosan after and before the radiation treatment for t hours, respectively

Irradiation by ultraviolet light, which possesses sufficient energy to cleavage covalent bonds, results in the formation of free radicals. The radicals produced can initiate further reactions such as degradation and/or recombination (Ramani and Ranganathaiah 2000). The photolysis of polymer leads mainly to a random scission of chain backbone. Two major degradation reactions, i.e., the random homolytic scission of main-chain carbon–carbon bonds and photolysis or photodissociation of side groups can occur. Oligomer formed after irradiation is mainly caused by chain depolymerization after photolytic scission. Similar to the γ-irradiation degradation, degradation rate in ultraviolet (UV) light condition decreases with increasing polymer concentration (Figure 31.2). The M_w of the

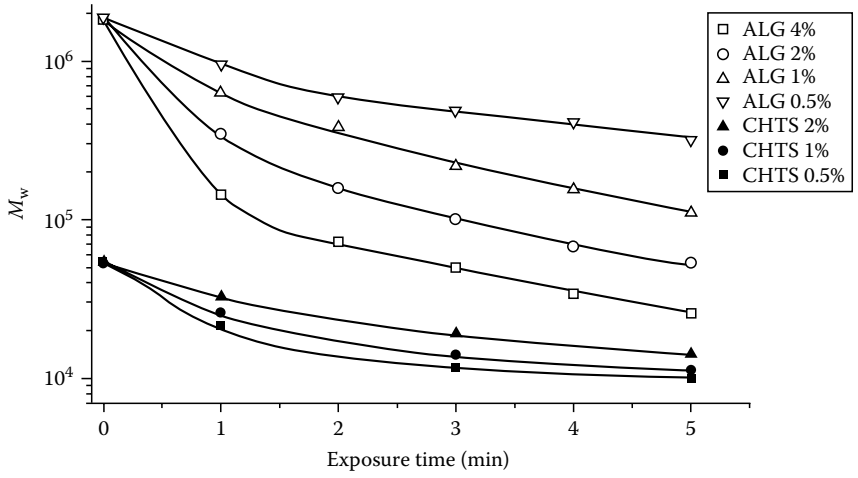


FIGURE 31.2 UV light degradation of sodium alginate and chitosan in aqueous solutions of different concentrations, at different UV light exposure times. (From Wasikiewicz, J.M. et al., *Radiat. Phys. Chem.*, 73, 287, 2005b. With permission.)

final degraded products of photolysis and radiolysis are at the same order, i.e., a few thousands. Although both the degradations are attributed to free radical reactions, the mechanism of radical creation is different (Ulanski and von Sonntag 2000). In γ-irradiation, the energy is absorbed mainly by water. Consequently, the macroradicals are formed during the reaction between the products of water radiolysis and polysaccharides, whereas in UV-irradiation the macroradicals are produced by the direct absorption of energy by the polysaccharide, and then result in the excitation and ionization of polysaccharide.

In the cases of aqueous solutions of chitosan (10 mL) irradiated in the air atmosphere by γ-ray at a dose from 0.5 to 200 kGy and by UV light irradiation from An EYE UV Cleaner working at 1 kW power and nominal efficiency 90% within 15 min, the degradation rate constant for chitosan in UV light irradiation is higher than that in γ-irradiation. However, taking into consideration the consummated energy, degradation by γ-irradiation is more efficient than UV light irradiation. Fourier-transform infrared (FTIR) and UV spectrometry measurements confirmed that the degradation proceeds by the breakage of glycoside bonds, which is caused by a radical scission mechanism (Wasikiewicz et al. 2005b).

31.2.1.3 Degradation in the H_2O_2 Aqueous Solution

The radiation-induced degradation mechanism of chitosan in the aqueous H_2O_2 solution is more complicated than that in solid state. Besides the reactions in solid state, the primary reactions might occur as follows (reactions (31.11) through (31.14)):

$$^{\bullet}H + H_2O_2 \longrightarrow H_2O + {}^{\bullet}OH \tag{31.11}$$

$$^{\bullet}OH + H_2O_2 \longrightarrow H_2O + HO_2^{\bullet} \tag{31.12}$$

$$e_{aq}^{-} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^{-} \tag{31.13}$$

$$H_2O_2 \xrightarrow{\gamma\text{-ray}} 2{}^{\bullet}OH \tag{31.14}$$

Chitosan could react with the $^{\bullet}OH$ radicals, which are derived from the radiolysis of water and H_2O_2, in which the reduction species $^{\bullet}H$ and e_{aq}^{-} are transformed into $^{\bullet}OH$. The results suggest that the addition of a small amount of H_2O_2 could greatly increase the degradation of chitosan with γ-irradiation.

The relationship among H_2O_2 content, dose, and M_η of chitosan is also studied (Kang et al. 2007, Lu et al. 2004). As Figure 31.3 shows, for irradiated chitosan without H_2O_2 as sensitizer, its M_η decreases gradually with the absorbed dose, and drops to 2.2×10^5 at 72 kGy. At a concentration of 38.2 wt% H_2O_2, a rapid drop of M_η is observed at 0.4 kGy, and then it decreased gradually up to 2 kGy, where the M_η reduced to 2.7×10^4. At low H_2O_2 concentration, the relationship between M_η and dose is similar to that at high H_2O_2 concentration. It suggests that the 1,4-glycosidic bonds of chitosan could be broken by radiation-induced scissions and oxidation of $^{\bullet}OH$ radicals through the decomposition of H_2O_2 and water, causing an obvious reduction in the M_η of polymer. Hence, the radiation treatment on chitosan in the presence of H_2O_2 could reduce its molecular weight effectively. When the degradation reaches a certain extent, the short molecular chain and few free amino groups will cause difficulty in further degradation, and, consequently, M_η of polymer reaches a minimum value.

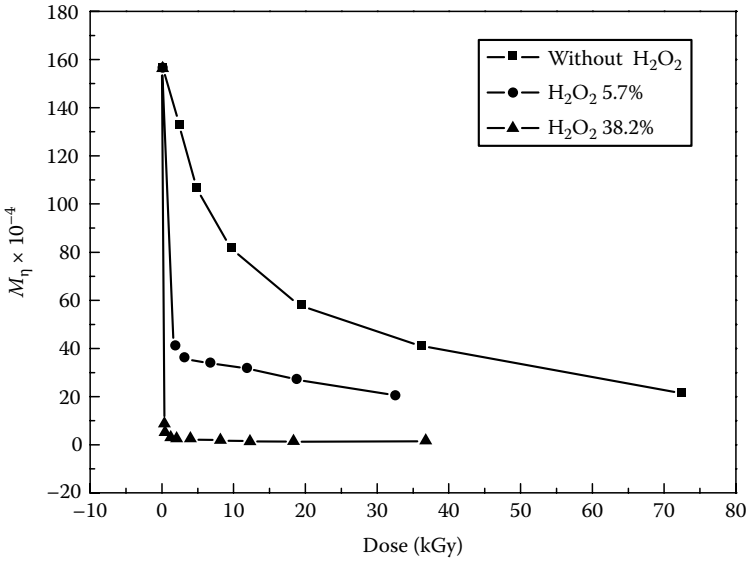


FIGURE 31.3 Effect of absorbed dose on the M_η of chitosan.

The degree of acetylation (DA) and the degree of deacetylation (DDA) of chitosan can be determined and calculated based on the FTIR spectra and equations described as follows (Brugnerotto et al. 2001):

$$\frac{A_{1320}}{A_{1420}} = 0.3822 + 0.03133\text{DA} \tag{31.15}$$

$$\text{DDA} = (1 - \text{DA}) \times 100\% \tag{31.16}$$

where A_{1320} and A_{1420} are the intensities of absorption bands at 1320 and 1420 cm^{-1}, respectively. The peaks of chitosan that appear at 3450, 1650, 1375, and 1020 cm^{-1} can be attributed to the hydroxyl, carbonyl or carboxyl, methyl, and C–O–C groups, respectively (Carlos et al. 1993). No obvious change is observed in the spectra of unirradiated and irradiated chitosan in the absence of H_2O_2. For the products irradiated in 10% H_2O_2, the peak at 1650 cm^{-1} increased remarkably with the increasing dose, which manifested the formation of carbonyl or carboxyl group after degradation. A simultaneous increase of peak at 1375 cm^{-1} indicated the formation of methyl group after ring-opening reaction. However, for the products irradiated in 30% H_2O_2, the peak at 1650 cm^{-1} assigned to carbonyl group is very weak at the same dose. Furthermore, the DDA values increases for irradiated chitosan with H_2O_2 (Figure 31.4).

The crystalline state of chitosan and radiation-degraded chitosan can be investigated by XRD. As Figure 31.5 shows, the XRD spectra of irradiated chitosan without H_2O_2 has a similar crystalline state to that of unirradiated chitosan ($2\theta = 10.8°$ and 20.1°), the irradiated sample in the presence of H_2O_2 has three peaks ($2\theta = 10.2°$, 19.8°, and 21.6°). This indicates that the radiation-degraded chitosan accelerated by H_2O_2 has more perfect crystalline structure than unirradiated chitosan. Chitosan chains with low molecular weight have higher mobility than chitosan with high molecular weight, which is easier to array regularly to form more perfect crystalline structure. In addition, the increase of DDA could improve the crystalline degree.

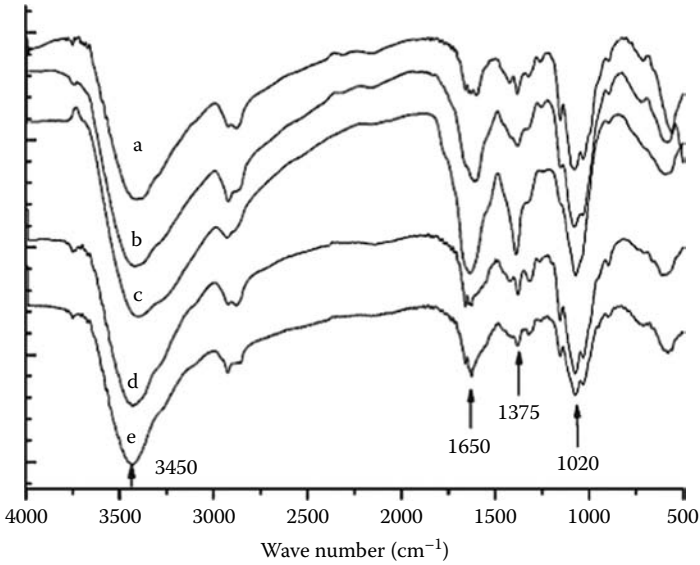


FIGURE 31.4 FTIR spectra of chitosan: initial chitosan (a); after irradiation in 10% H_2O_2 at the dose of 50 kGy (b) and 100 kGy (c); after irradiation in 30% H_2O_2 at the dose of 50 kGy (d) and 100 kGy (e). (From Kang, B. et al., *Polym. Degrad. Stabil.*, 92, 359, 2007. With permission.)

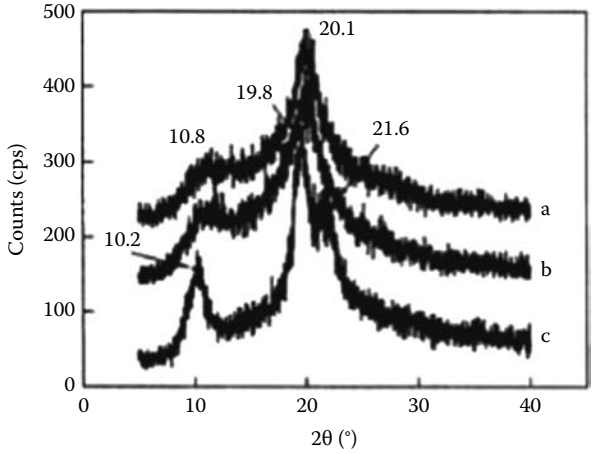


FIGURE 31.5 XRD patterns of initial and degraded chitosan: initial chitosan (a), irradiated chitosan without H_2O_2 (b), irradiated chitosan with 38.2 wt% H_2O_2 (c).

The elemental analysis of chitosan samples before and after degradation manifested that the mass ratio of N/C and H/C reduced during degradation, which confirmed the loss of nitrogen during degradation in the presence of 10% H_2O_2. However, when the concentration of H_2O_2 was 30%, the N/C mass ratio of chitosan increased compared to the initial sample due to the decrease in carbon contents (Kang et al. 2007). The results of UV–vis and FTIR spectra indicated that the formation of carbonyl and carboxyl groups coexisted with the degradation of chitosan when the concentration of H_2O_2 was 10%, but a different situation was observed in 30% H_2O_2. The crystallinity of chitosan decreased

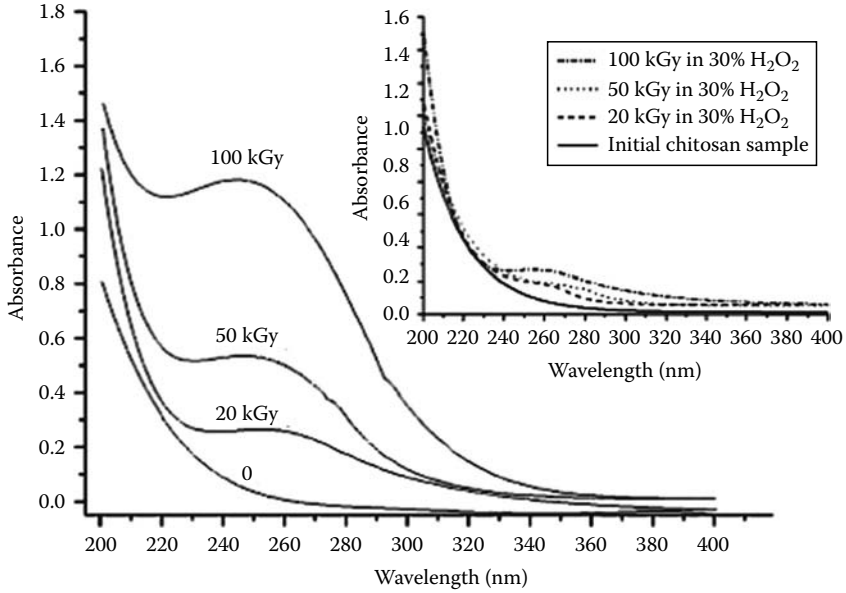


FIGURE 31.6 UV–vis spectra of original and degraded chitosans irradiated in 10% H_2O_2. Inset: irradiated in 30% H_2O_2. (From Kang, B. et al., *Polym. Degrad. Stabil.*, 92, 359, 2007. With permission.)

remarkably with degradation and the crystalline state of water-soluble product was entirely different from that of water-insoluble product.

The UV spectra of chitosan irradiated with various doses in the presence of 10% H_2O_2 were monitored in Figure 31.6. A new absorption band between 250 and 280 nm was observed and the peak intensity increased with increasing dose. This peak can be ascribed to carbon–oxygen double bonds formed after the main chain scission of chitosan and hydrogen abstraction reaction followed by the ring opening (Ulanski and Rosiak 1992). The UV spectra of chitosan irradiated in 30% H_2O_2 show that the peaks between 250 and 280 nm assigned to the double bonds are very weak. It is assumed that the active oxygen atoms, as super powerful oxidizing species, were formed under radiation and destroyed carbonyl or carboxyl groups in a high concentration but not in low concentration of H_2O_2.

Furthermore, a synergetic effect that combines UV light irradiation and hydrogen peroxide oxidation exists during the degradation of chitosan. When the initial chitosan was exposed to UV irradiation for 30 and 180 min, the decrement in viscosity was 17.2% and 26.4%, respectively. While under the oxidation of hydrogen peroxide for 30 and 180 min, the decrement in viscosity was 20.6% and 63.7%, respectively. It is interesting to note that when combined with UV radiation and hydrogen peroxide oxidation, the decrement in viscosity increases to 84.3% and 99.2%, which is higher than the summation of those numerical values obtained individually by UV radiation or hydrogen peroxide processes. In other words, synergetic effects are operative in the degradation of chitosan with UV light and hydrogen peroxide. The optimum reaction conditions were obtained by an orthogonal test using hydrogen peroxide 2% (w/v), chitosan 2% (w/v), acetic acid 1% (w/v), and an irradiation time of 30 min. During degradation, chitosan oligomers retained the backbone of the chitosan macromolecular structure; it is the breaking of the C–O–C glycoside bond that led to the chain scission and the formation of carbonyl groups. Therefore, these degradation strategies are feasible, convenient, and potentially applicable to prepare low M_w chitosan (Wang et al. 2005).

31.2.2 Radiation-Induced Degradation of CM-Chitosan

31.2.2.1 Degradation in Solid State

CM-chitosan is a kind of carboxymethylated derivative of chitosan, which is prepared by the chemical reactions of chitosan and monochloroacetic acid. It contains –COOH and $-NH_2$ groups in the molecular structure. Being a soluble chitosan derivative in both acidic and basic physiological media, it might be a better candidate than chitosan for studying the mechanism of degradation reaction of chitosan derivatives.

The viscosity of CM-chitosan can be determined by the viscometric method with an Ubbelohde capillary viscometer. A One-Point method equation was used to calculate the intrinsic viscosity (Fan et al. 2002):

$$[\eta] = \frac{4\eta_{sp}^{1.02} \ln \eta_r}{C^{1.01}(3\eta_{sp} + \ln \eta_r)} \tag{31.17}$$

where

$[\eta]$ is intrinsic viscosity
η_r is relative viscosity
η_{sp} is specific viscosity
C is the concentration of CM-chitosan in 0.1 mol/L sodium chloride aqueous solution

The viscosity average molecular weight of CM-chitosan can be calculated by Mark–Houwink equation with $K = 7.92 \times 10^{-5}$, $\alpha = 1.00$ (Zhao et al. 2002).

G_d is defined as radiation chemical yield which represents the number of radiolysis events caused by the absorption of 100 eV of radiation energy. G_d expresses the degradation susceptibility of the polymer during radiation and can be calculated by Equation 31.18:

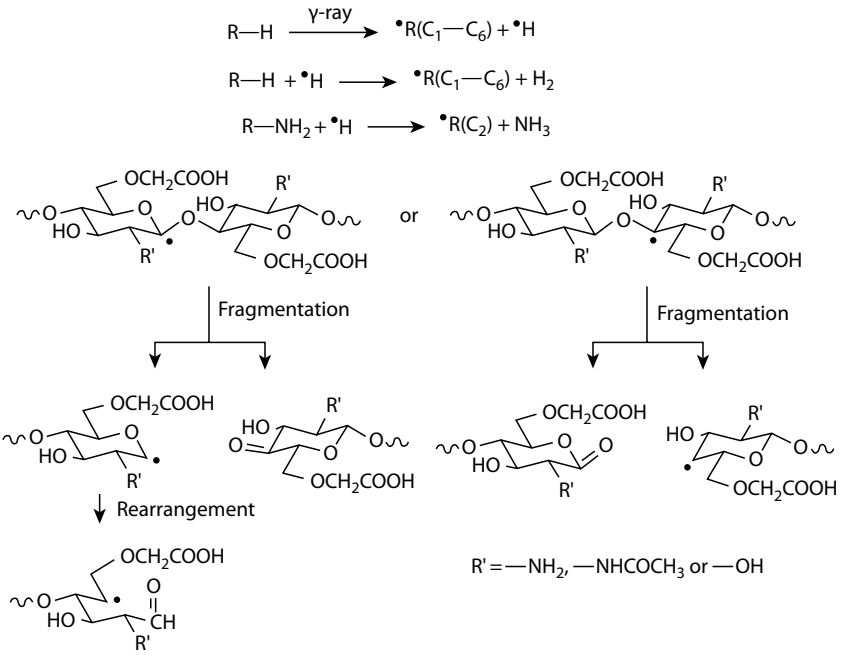
$$\left(\frac{1}{M_n} - \frac{1}{M_{n0}}\right) = \frac{G_d \times 6.24 \times 10^{16}}{N_A} \times D \tag{31.18}$$

where

M_{n0}, M_n are number-average molecular weights of the polymer before and after degradation respectively
N_A is Avogadro's number
D is dose

When $\alpha = 1$, $M_\eta = 2\ M_n$. G_d of CM-chitosan in solid state calculated by the Charlesby–Pinner equation is 0.49, which means that CM-chitosan has good stability even when the absorbed dose reaches the sterilization dose (25 kGy). Therefore, it can be sterilized by radiation that is promising for biomedical application.

In the UV spectra of degraded CM-chitosan in solid state, new absorption band at approximate 260 nm appeared and the peak intensity increased with increasing absorbed dose, which resulted from the formation of carbonyl and carboxyl groups or the elimination of amino groups in the radiation process. The same evidence can be obtained in the FTIR spectra of original and degraded CM-chitosan with 20 kGy dose. XRD patterns show that the peak intensities of CM-chitosan increased with the increasing dose, which means that the degradation mostly occurred in the amorphous region of CM-chitosan. According to the above-mentioned results, the degradation mechanism of CM-chitosan in solid state is proposed as Scheme 31.1 (Huang et al. 2007a).



SCHEME 31.1 The degradation mechanism of CM-chitosan in solid state during irradiation. (From Huang, L. et al., *Radiat. Phys. Chem*., 76, 1679, 2007a. With permission.)

31.2.2.2 Degradation in Diluted Aqueous Solution

The G_d of radiation-induced degradation of CM-chitosan in various conditions was listed in Table 31.1.

In the CM-chitosan solutions that contained 0.02 mol/L H_2O_2 (condition c) or 2.5×10^{-2} mol/L N_2O (condition b), G_d were 280% and 150% of that in condition a (N_2-saturated). In condition d, because 0.76 mol/L isopropanol was added into the CM-chitosan solution, G_d decreased to 5% of that in condition a. Similar to the degradation mechanism of chitosan in aqueous solution, the radiation energy of γ-ray is absorbed mainly by water in dilute CM-chitosan aqueous solutions, and the direct effect of radiation on CM-chitosan can be neglected. The radiation chemical yield of reactive species released in the radiolysis of water are constant in the wide range of pH. In condition a, CM-chitosan aqueous solution was radiated with saturated N_2. The active species that resulted in

TABLE 31.1
G_d of Radiation Degradation of CM-Chitosan in Different Conditions

Sample	Condition	G_d	r	Additives and Ambience
CM-chitosan	a	62.9	0.999	N_2-saturated, pH = 9.8
	b	95.9	0.996	N_2O-saturated, pH = 9.8
	c	176	0.997	N_2-saturated, 0.02 mol/L H_2O_2, pH = 9.8
	d	3.19	0.999	N_2-saturated, 0.76 mol/L isopropanol, pH = 9.8
	e	46.3	0.999	N_2-saturated, pH = 7.9
	f	20.3	0.986	N_2-saturated, pH = 1.1

Source: Huang, L. et al., *Carbohydr. Polym*., 67, 305, 2007b. With permission.

the degradation of CM-chitosan were •OH, •H, and e_{aq}^-, and the effect of other reactive transients can be neglected. In condition b, e_{aq}^- can be readily converted into •OH radicals (reaction 31.19) and G_d increased to 150% of that in condition a.

$$e_{aq}^- + N_2O + H_2O \rightarrow {}^{\bullet}OH + N_2 + OH^- \tag{31.19}$$

Furthermore, the radiolysis of H_2O_2 forms •OH by reaction (31.14) directly in condition c, and e_{aq}^- can also react with H_2O_2 to produce •OH by reaction (31.13). A low concentration of H_2O_2 (0.02 mol/L) can make G_d increased by 280%.

$$e_{aq}^- + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- \tag{31.13}$$

$$H_2O_2 \rightarrow 2{}^{\bullet}OH \tag{31.14}$$

Isopropanol is the scavenger of •OH and •H. It can react with •H or •OH radicals and form isopropanol radicals, which are inert for polymers (reactions (31.20) and (31.21)) (Zhai et al. 2004a,b). In condition d, 0.76 mol/L isopropanol-induced G_d decreased to 5%.

$$(CH_3)_2CHOH + {}^{\bullet}H \rightarrow (CH_3)_2\dot{C}OH + H_2 \tag{31.20}$$

$$(CH_3)_2CHOH + {}^{\bullet}OH \rightarrow (CH_3)_2\dot{C}OH + H_2O \tag{31.21}$$

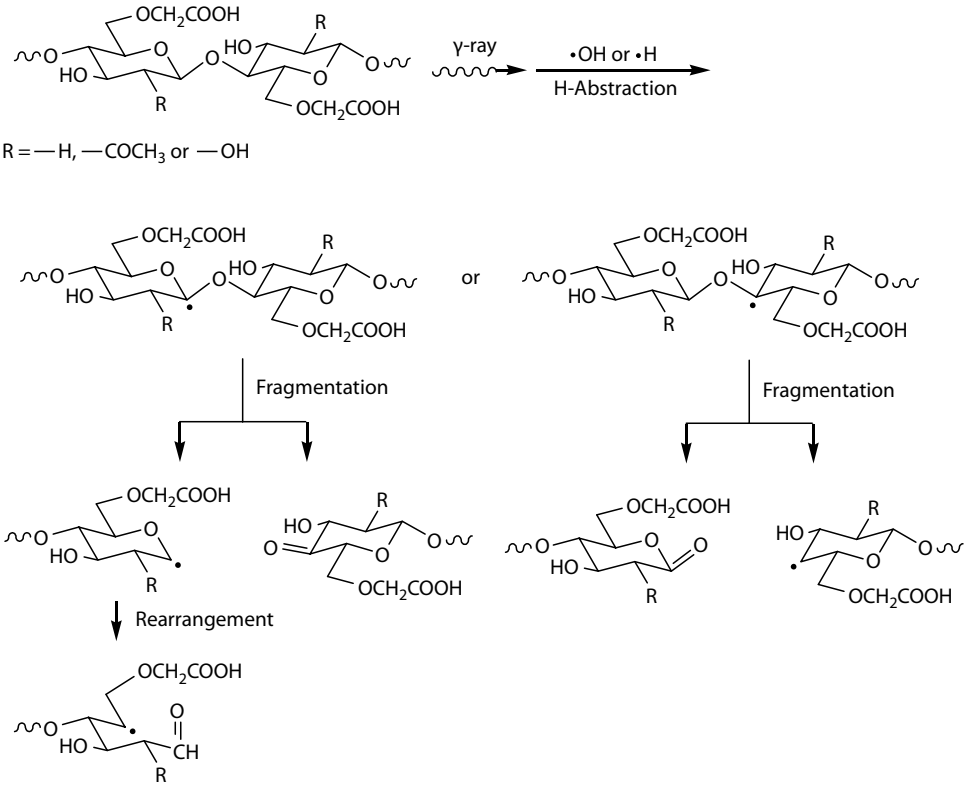
From the above-mentioned experimental results, we understand that e_{aq}^- can hardly initiate the degradation of CM-chitosan immediately, and both •H and •OH are the main reactive radicals for the H-abstraction reactions of CM-chitosan in dilute CM-chitosan aqueous solution.

In conditions a, e, and f, the reactive transients were the products of water radiolysis. Their radiation chemical yields may be diverse in a neutral or alkaline solution, but the difference is often neglected. Therefore, the radiation chemical yields of these transients such as •H, •OH, and e_{aq}^- are constant in the wide range of pH. However, e_{aq}^- can also convert to •H quantitatively in acid solution such as in condition f (reaction (31.22)).

$$e_{aq}^- + H^+ \rightarrow {}^{\bullet}H \tag{31.22}$$

e_{aq}^- converts into •H can accelerate the degradation of CM-chitosan, which means that in an acidic condition, there are more •H to initiate the degradation of CM-chitosan. However, a contrary result was obtained in this work. It suggested that the change of the conformation of CM-chitosan molecules with pH is a main factor that influenced the degradation of CM-chitosan. The action of •H can be neglected in acid solution. In fact, the radiation-chemical yield of •H comprises less than 10% (even pH = 2, less than 20%) of water radiolysis transients. When compared to •OH, •H reacts with polymers at one order of magnitude lower rate constant, and its role for degradation can be neglected, thus in the radiation system of dilute CM-chitosan aqueous solution, •OH radicals played the most important role for the degradation of CM-chitosan.

In a comparison of CM-chitosan and CM-chitin, which have similar DS, molecular weight, and different DDA (84.0% for CM-chitosan and 31.4% for CM-chitin), CM-chitosan decreased more quickly than CM-chitin. It is because that •OH is a strong electrophilic reagent and H-abstraction



SCHEME 31.2 The possible mechanism of main chain scission of CM-chitosan during radiation. (From Huang, L. et al., *Carbohydr. Polym*., 67, 305, 2007b. With permission.)

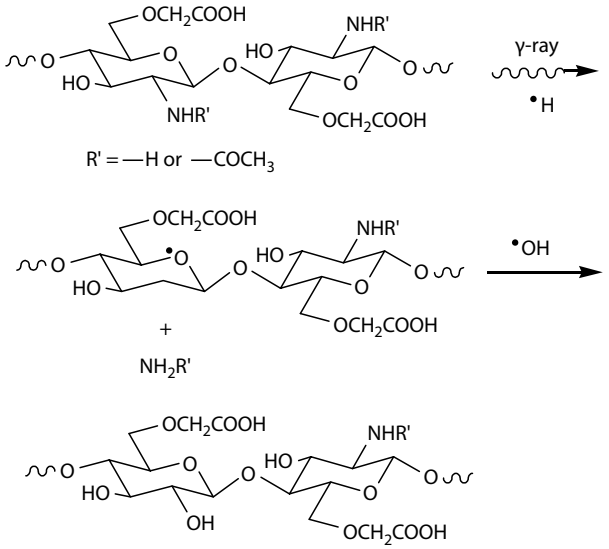
reaction mostly occurred at the position with a high density of electron, which is the reason that CM-chitosan, with a high DDA and high density of electron, degraded much faster than CM-chitin.

FTIR and UV spectra showed that the main backbone chain structure of CM-chitosan remained and some carbonyl/carboxyl groups were formed during the degradation. The elimination of partial amino groups during radiation was also identified by the increase of C/N contents in element analysis data. The possible mechanism of degradation and elimination of amino groups during radiation process are shown in Schemes 31.2 and 31.3 (Huang et al. 2007b).

31.2.2.3 Laser Photolysis

Laser photolysis experiments on carboxymethylated chitin derivatives, such as CM-chitin and CM-chitosan, in aqueous solution by a 248 nm excimer laser was carrier out for the first time (Zhai et al. 2004b). The transient absorption spectra of photolyzed CM-chitin or CM-chitosan solutions revealed a strong band with the maximum at 720 nm, which was assigned to the e_{aq}^-. In the presence of argon, the e_{aq}^- decays by reacting with CM-chitin or CM-chitosan, and the rate constants are $(6.1 \pm 0.1) \times 10^7$ and $(3.7 \pm 0.1) \times 10^7$ M^{-1} s^{-1}, respectively. Long-lived radicals with relatively weak absorption intensity were detected in the near-UV region. The absorption band was not notably characteristic and showed only an increasing absorption toward shorter wavelengths. It is similar to the signal of •CM-chitin or •CM-chitosan macroradicals formed by e_{aq}^- + CM-chitin or CM-chitosan reaction.

The reaction of •OH or $•SO_4^-$ radicals with CM-chitin and CM-chitosan were investigated in aqueous solutions using a laser photolysis technique (Zhai et al. 2004a). The rate constants of the reactions between •OH or $•SO_4^-$ radicals with CM-chitosan are always higher than those for



SCHEME 31.3 The possible mechanism of amino groups eliminated from CM-chitosan during radiation. (From Huang, L. et al., *Carbohydr. Polym*., 67, 305, 2007b. With permission.)

CM-chitin, indicating that the amino group could increase the reactivity of CM-chitin derivatives. The reaction rate of CM-chitin and CM-chitosan with •OH radical was found to decrease at lower pH when polymers chains tend to the coiled conformation. In comparison, the rate constant of the reaction of $^{\bullet}SO_4^-$ radicals with CM-chitin or CM-chitosan decreased with pH, indicating that CM-chitin or CM-chitosan has a higher reactivity with the $^{\bullet}SO_4^-$ radical at low pH due to the protonation of the amino group.

31.2.3 Applications of Degraded Chitosan and Its Derivatives

31.2.3.1 Antioxidant Activity

Chitosan oligomers with different molecular weights could be prepared by oxidative degradation with H_2O_2, or the microwave radiation degradation in the presence of H_2O_2. Both methods were effective to prepare chitosan oligomers from common chitosan (8.5×10^5 Da). The degradation process of chitosan will be accelerated with the aid of microwave. The antioxidant activity of chitosan oligomers was evaluated as radical scavengers against superoxide anion and hydroxyl radical by the application of flow injection chemiluminescence technology. Chitosan oligomers A, B, C, and D (2,300, 3,270, 6,120, and 15,250 Da) had different antioxidant activity. Among the four chitosan oligomers, oligomer D had the lowest scavenging ability against superoxide anion and hydroxyl radicals. For superoxide anion scavenging, the 50% inhibition concentrations (IC_{50}s) of other three oligomers A, B, and C were 5.54, 8.11, and 12.15 mg/mL, respectively. And for hydroxyl radical scavenging the values were 0.4, 0.76, and 1.54 mg/mL, respectively. At the tested concentration range, the maximal inhibiting efficacy of A, B, C, and D were 89%, 75%, 74%, and 41% for superoxide anion, and 71%, 65%, 51%, and 7% for hydroxyl radical. These results indicated that chitosan oligomers with lower molecular weight had better antioxidant activity (Sun et al. 2007).

31.2.3.2 Antimicrobial Activity

The antimicrobial activity of irradiated chitosan was studied against *Escherichia coli* B/r. The irradiation of solid chitosan at 100 kGy was effective in increasing the activity, and inhibited the growth

of *E. coli* completely. The molecular weight of chitosan significantly decreased with the increase in the dose, whereas the relative surface charge of chitosan was decreased only 3% by 100 kGy irradiation. The antimicrobial activity assay of chitosan fractionated according to molecular weight showed that 1×10^5 to 3×10^5 fraction was most effective in suppressing the growth of *E. coli*. This fraction comprised only 8% of the 100 kGy irradiated chitosan. On the other hand, chitosan whose molecular weight was less than 1×10^5 lost its antimicrobial activity. The results show that low dose irradiation, specifically 100 kGy, of chitosan gives enough degradation to increase its antimicrobial activity as a result of molecular weight decreasing (Matsuhashi and Kume 1997).

31.2.3.3 Promotion of Plant Growth

Chitosan powder was irradiated with electron beam within the dose range of 20–250 kGy. The average molecular weight (M_w) of chitosan decreased remarkably with the increasing dose up to 200 kGy. There was no significant change in molecular weight for higher doses (Figure 31.7). The preliminary agricultural tests on spring rape seeds show that the highest growth yield was observed for chitosan (molecular weight 47,000 Da) in a concentration of 0.1 g/kg of seeds. The higher concentration did not affect plant's growth. The average growth of over-ground plant parts was about 16%–22%, the diameter of roots was about 11%–13%, and the mass of roots was about 51%–65% higher in comparison to the control (Figure 31.8). All the plants with chitosan had better developed roots and shoots, which indicated that chitosan has very positive impact on plants growth (Chmielewski et al. 2007).

The toxicity of vanadium (V) and the effect of chitosan have been investigated on soybean, rice, wheat, and barley. Wheat and barley were more sensitive to V than rice and soybean, and all the seedlings of these plants were damaged at 2.5 µg/mL V (in VCl_3). Figure 31.9 shows that chitosan, unirradiated or irradiated at a low dose of 20 kGy, induced the various levels of chlorosis in the shoots of seedlings. The chlorosis was decreased at 50 kGy and disappeared at 100 kGy. It is found that these damages were reduced by the application of radiation-degraded chitosan because radiation causes a significant decrease in viscosity, but the decrease in surface charge is small. Chitosan irradiated in 1% solution strongly affects the growth of wheat and rice plants. The recovery of growth and the reduction of V levels in seedlings were obtained by the treatments with 10–100 µg/mL chitosan irradiated at 70–200 kGy of γ-rays in 1% solution. The reductions of V and Fe contents in plants were due to the ability of chitosan to form chelate complexes with metals in solution. The result of

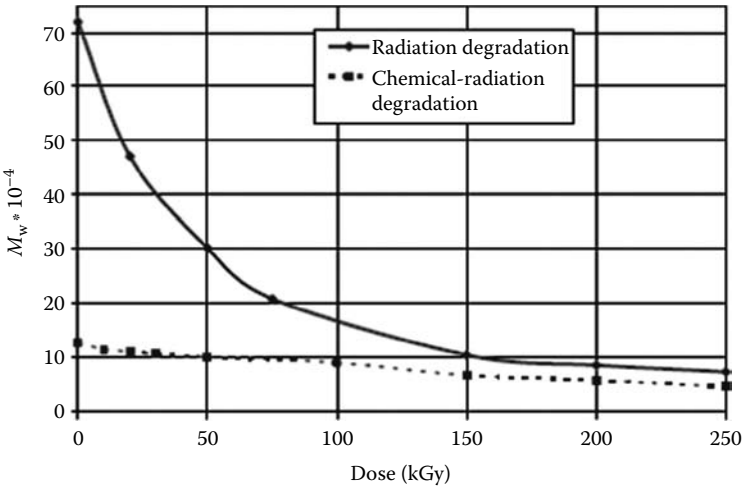


FIGURE 31.7 Radiation and chemical-radiation degradation of chitosan (hydrogen peroxide is added before irradiated by electron beam). (From Chmielewski, A.G. et al., *Radiat. Phys. Chem.*, 76, 1840, 2007. With permission.)

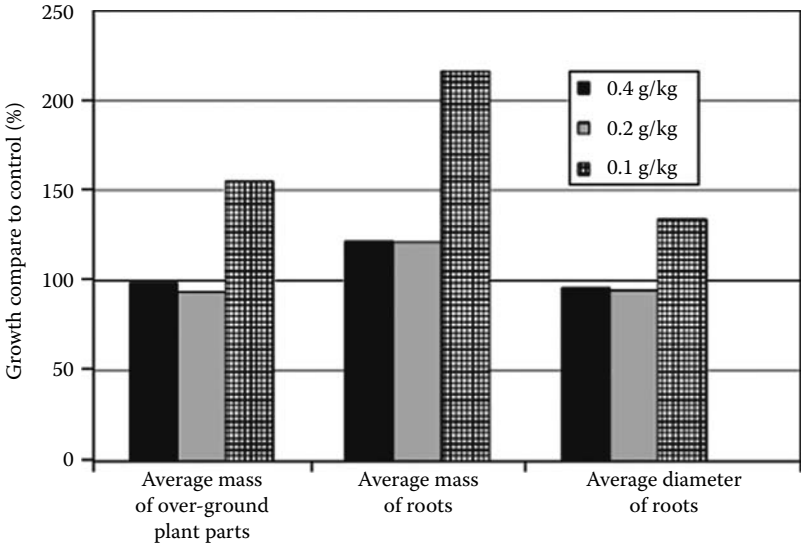


FIGURE 31.8 Growth of plant parts with chitosan in different concentration compared to the control (growth of control—100%). (From Chmielewski, A.G. et al., *Radiat. Phys. Chem*., 76, 1840, 2007. With permission.)

FIGURE 31.9 Effect of irradiated chitosan on rice growth. Rice was cultivated for 9 days in hydroponic solution with 100 μg/mL chitosan. From left (1) control (without chitosan); (2) with unirradiated chitosan; (3)–(5) 20, 50, and 100 kGy irradiated chitosan. (From Tham, L.X. et al., *Radiat. Phys. Chem*., 61, 171, 2001. With permission.)

bioimaging analyzer system (BAS) analysis shows that the absorption and transportation of ^{48}V to the leaf from the root was suppressed with irradiated chitosan. Therefore, it can be concluded that chitosan irradiated at suitable doses (ca. 100 kGy) is effective as plant-growth promoters and heavy metal eliminators in crop production (Tham et al. 2001).

31.2.3.4 Fat-Binding Ability

Chitosan with different molecular weight are obtained by ^{60}Co γ-ray irradiation in the solid condition and in the pH 3.0 ($HClO_4$) solutions, respectively. The influence of average molecular weight of chitosan on its fat-binding ability in vitro has been studied by using a biopharmaceutical model

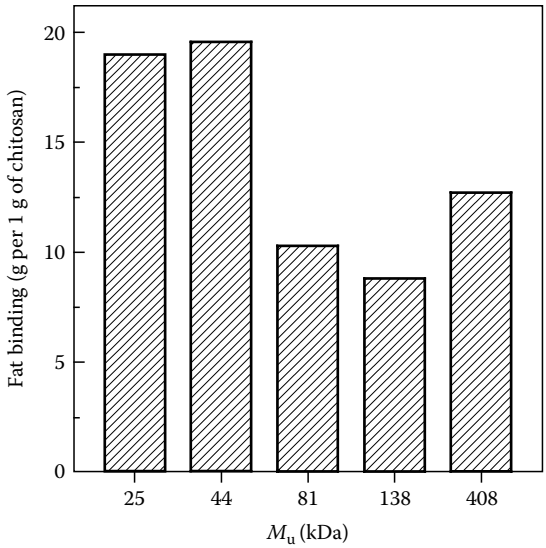


FIGURE 31.10 Amount of fat (g) bound by 1 g of chitosan as a function of weight-average molecular weight. (From Czechowska-Biskup, R. et al., *Nucl. Instrum. Methods Phys. Res. Sect. B*, 236, 383, 2005. With permission.)

of digestive tract. It is found that lowering the average molecular weight of chitosan in the tested range (ca. 25–400 kDa) leads to an increase in the fat-binding ability of chitosan. One gram of modified chitosan may bind up to 20 g of fat (Figure 31.10). This may be of practical importance, since it may allow reducing the daily dose of chitosan to obtain the same dietary effect (Czechowska-Biskup et al. 2005).

31.2.3.5 Natural Oligosaccharides-Derived Room Temperature Ionic Liquids

Natural oligosaccharides-derived room temperature ionic liquids (RTILs) were prepared from 1-ethyl-3-methylimidazolium hydroxide (EMIM · OH) and CM-chitosan by acid–base neutralization reaction (Huang et al. 2008). Low-molecular-weight CM-chitosan was prepared by γ-ray irradiation. The structure of RTILs was identified by UV, FTIR, and ^{1}H NMR. At 25°C, the ionic conductivities of EMIM · CM-chitosan ionic liquids with different molecular weight CM-chitosan (6×10^2, 8×10^2, and 1×10^3 Da) were 1.9×10^{-3}, 1.2×10^{-3}, and 7.5×10^{-4} S/cm, respectively (Figure 31.11). These EMIM · CM-chitosan ionic liquids exhibited good ionic conductivity and thermal stability, as well as low glass transition temperature, implying their potential wide applications in direct electrochemistry, biosensors and biocatalysis.

31.3 RADIATION-INDUCED CROSS-LINKING AND THEIR APPLICATIONS

Chitosan and its derivatives are typical radiation-degraded polymers (Chapiro 1962, Huang et al. 2007a, Ulanski and Rosiak 1992, Zaikov and Sharpatyi 2006, Zhao and Mitomo 2008). However, very recently, in several studies conducted, it is found that some hydrophilic chitosan derivatives such as CM-chitin, CM-chitosan, and DHP-chitosan undergo cross-linking to form hydrogels when exposed to high concentrated aqueous solution (more than 10%, paste-like state) (Wasikiewicz et al. 2006, Yoshii et al. 2003, Zhao et al. 2003, 2008b, Zhao and Mitomo 2008, 2009) in γ-ray or electron beam. Here, high concentrated means that the solutions have a tixotropic paste-like property, which is distinguished from the viscous solutions that exhibited slow gravity flow.

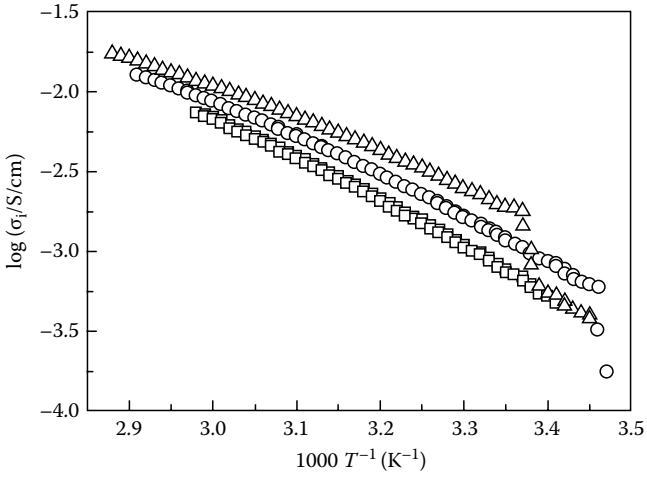


FIGURE 31.11 Temperature dependence of the ionic conductivities of EMIM·CM-chitosan ionic liquids with different molecular weight CM-chitosan: 1×10^3 Da (□), 8×10^2 Da (○), 6×10^2 Da (△). (From Huang, L. et al., *Carbohydr. Polym*., 71, 690, 2008. With permission.)

From these findings, a green fabrication approach to prepare novel hydrogel materials from chitosan derivatives without any biofunctional cross-linking agents was established. Compared to other methods, radiation-induced cross-linking can occur in aqueous polymer solutions at room temperature without any initiator or cross-linker. Moreover, ionizing radiation usually allows a combination of the synthesis and sterilization of polymeric materials in a single step. Therefore, radiation method is an excellent tool in the fabrication of materials for biomedical applications. Cross-linking behavior and some basic properties of cross-linked products (hydrogels) are described in this chapter. Some applications of these hydrogel materials in environment field are also introduced here.

31.3.1 Cross-Linking of CM-Chitin, CM-Chitosan, and DHP-Chitosan

CM-chitin and CM-chitosan are commercially available water-soluble chitosan derivatives (Hayes 1986, Kumar and Majeti 2000). As described in the degradation portion of CM-chitin, CM-chitosan undergoes radiation degradation in solid state and diluted solution (below 10%). Radiation cross-linking occurs only in high-concentration solutions, namely, "paste-like status" (Wasikiewicz et al. 2006, Yoshii et al. 2003, Zhao et al. 2003). The concentration of CM-chitin and CM-chitosan in aqueous solution remarkably affected the course of cross-linking reaction. The effects of the concentration of CM-chitosan in aqueous solution on the gel fraction is shown in Figure 31.12. Gel fraction increased sharply just after exceeding the gelation point and then leveled off. A high concentration, e.g., 30%–40% for CM-chitosan, is favorable for cross-linking. A similar situation is also observed for CM-chitin. The paste-like condition (10%~40%) could provide suitable mobility and distance for the macroradicals to form intermolecular cross-linking. However, for a higher concentration (i.e., 50%), lower gel fraction was obtained due to the micro-phase separation of substance and solvent, in other words, it is difficult to obtain homogeneous polymer solutions. Therefore, an ideal condition for radiation cross-linking is that the polymer should be fully mixed with water to prepare homogeneous solutions.

Similar with CM-chitosan, DHP-chitosan is also a highly hydrophilic polymer, with good processability, biocompatibility, antibacterial activity, biodegradability, and low costs (Kobayashi et al. 2005, Maeda et al. 1997). However, it is hardly soluble in water because of the strong intermolecular hydrogen bonding. DHP-chitosan can be dissolved in water by adding diluted acid as a subversive of hydrogen bonding. The lactic acid aqueous solution of 2% is used to dissolve the

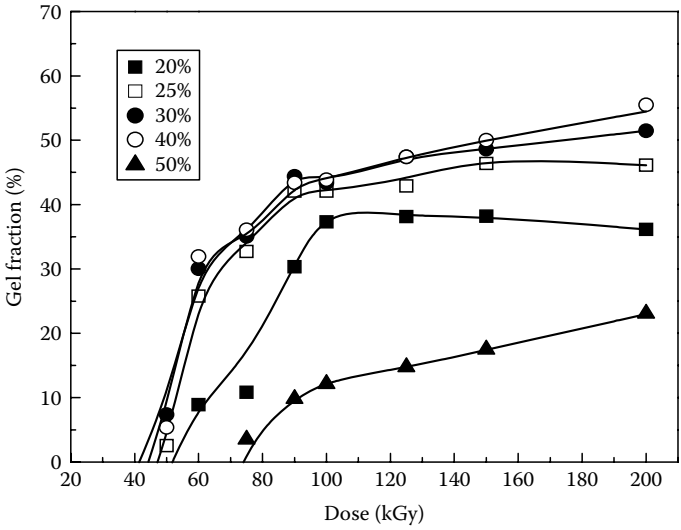


FIGURE 31.12 Effect of concentration on cross-linking of CM-chitosan (irradiation in vacuum). (From Zhao, L. et al., *Carbohydr. Polym*., 51, 169, 2003. With permission.)

DHP-chitosan in this case. A high concentrated DHP-chitosan sample (more than 10%) can be mixed homogeneously.

DHP-chitosan underwent degradation at solid state and diluted solution (below 10%) (Zhao and Mitomo 2008). When DHP-chitosan is irradiated at moderate or high concentration, the intermolecular cross-linking of DPH-chitosan prevails and the gel is formed (Zhao and Mitomo 2008, 2009). The cross-linking and degradation conditions for DHP-chitosan are similar to that of CM-chitin and CM-chitosan. The gel fraction increases with absorbed dose, sharply at the beginning of gelation, and levels off asymptotically to the maximum value. The highest gel fraction (ca. 50%) was obtained for a concentration of 40 wt%. In the case of higher concentration (i.e., 50%), as mentioned in CM-chitosan portion, it is difficult to obtain homogeneously dispersed polymer in the whole volume of sample. As a result, low gel fraction was obtained due to the heterogeneous status of the solution. Figure 31.13 elucidated the effect of irradiation at 100 kGy in various conditions.

For the radiation degradation of polysaccharide derivatives, a low concentration is favorable for yielding low-molecular-weight products; however, a high concentration in solution is favorable for the radiation cross-linking of polysaccharides. It is considered that two factors enhance the cross-linking in high-concentrated solution state. One is the sufficient mobility and another is the mutual vicinity of macroradicals. In addition, as mentioned above, the presence of water also enhances the yield of macroradicals. In the case of DHP-chitosan, an attempt has been made to investigate the influence of the high-concentrated state on irradiation processing using x-ray diffraction (Figure 31.14). The cross-linked DHP-chitosan and DHP-chitosan paste are more amorphous than the original DHP-chitosan. Crystalline peaks decreased greatly after lactic acid treatment in paste condition, implying the decrease of intermolecular hydrogen bonding, while crystalline peaks decreased greatly after cross-linking, indicating the formation of gel network structure. The decrease of intermolecular hydrogen bonding could result in the sufficient mobility in high-concentrated solution state, and for this reason, DHP-chitosan can be induced for cross-linking by irradiation.

The radiation effect of chitosan derivatives can be summarized as follows: solid state and low-concentrated solution state result in degradation, while high-concentrated solution state is favorable for cross-linking. The irradiation effect of chitosan derivatives in different conditions are proposed in Scheme 31.4.

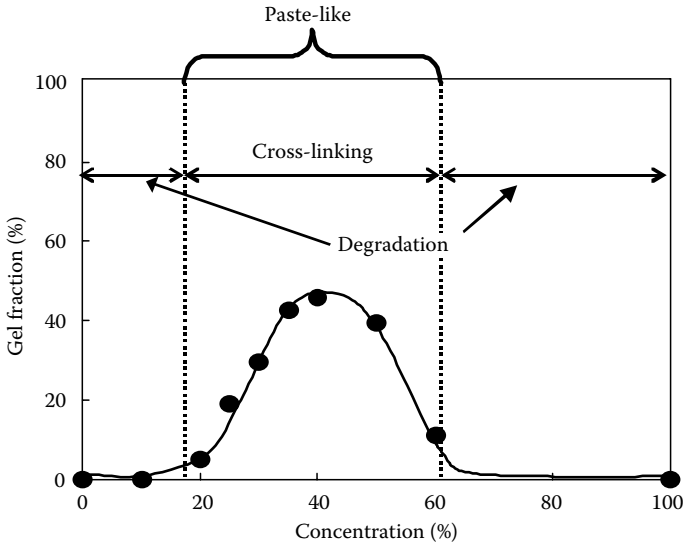


FIGURE 31.13 Radiation-induced degradation and cross-linking of DHP-chitosan in various conditions (irradiated at 100 kGy). (From Zhao, L. and Mitomo, H., *Carbohydr. Polym.*, 76, 314, 2009. With permission.)

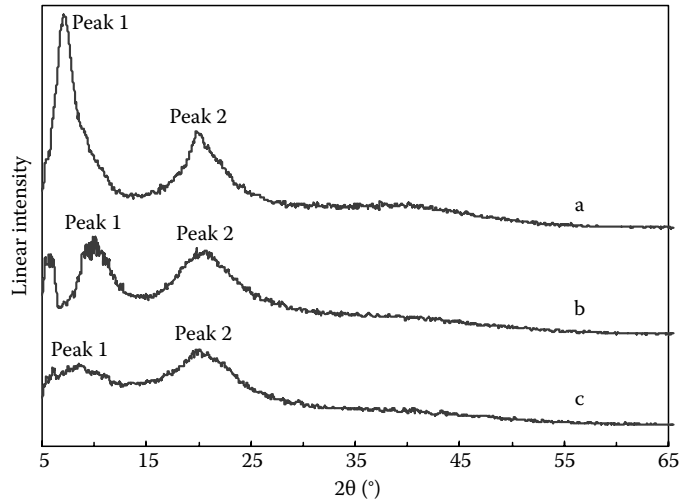
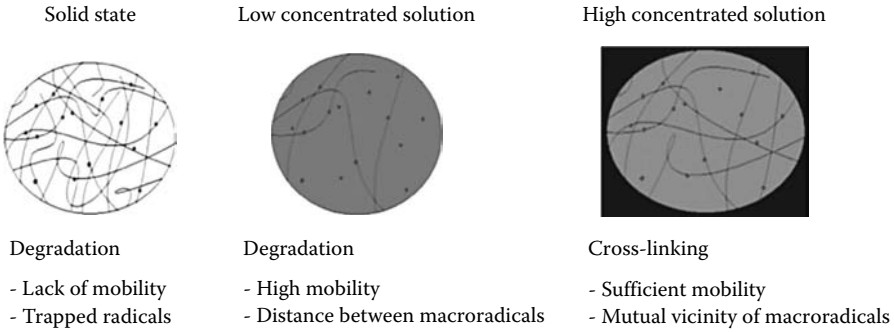


FIGURE 31.14 X-ray diffraction patterns of (a) original DHP-chitosan, (b) DHP-chitosan paste, (c) cross-linked DHP-chitosan. (From Zhao, L. and Mitomo, H., *Polym. Degrad. Stabil.*, 93, 1607, 2008. With permission.)

31.3.2 Characterization of the Gelation Procedure

The cross-linking behavior of chitosan derivatives can be evaluated by calculating some important values related to the formation of hydrogels, such as D_g, $p_0/q_{0,}$ according to Charlesby–Rosiak equation (Olejniczak et al. 1991):

$$s + \sqrt{s} = \frac{p_0}{q_0} + \left(2 - \frac{p_0}{q_0}\right)\frac{D_v + D_g}{D_v + D} \tag{31.23}$$



SCHEME 31.4 Irradiation effect of chitosan derivatives in different conditions (the points represent radicals induced by irradiation). (From Zhao, L. and Mitomo, H., *Polym. Degrad. Stabil.*, 93, 1607, 2008. With permission.)

$$G(x) = \frac{4.8 \times 10^5}{\overline{M_{w0}} \times D_g} \tag{31.24}$$

where

s is sol fraction

p_0 is degradation density—represents the average number of main chain scission per monomer unit and per unit dose

q_0 is cross-linking density—represents the proportion of monomer units cross-linked per unit dose

D is the absorbed dose

D_v is the virtual dose—means a critical dose to make the weight-average and number-average molecular weight equal to 2

M_{w0} is the initial weight-average molecular weight of polymer

The gelation dose (D_g) represents the minimum required dose to initiate gelation process. p_0/q_0 is the parameter that allows the determination of the final results of irradiation and is equal to half of the scission yield/cross-linking yield ratio [$0.5 \times G_{(S)}/G_{(x)}$]. If p_0/q_0 is lower, cross-linking occurs more efficiently.

The data concerning the tendency of cross-linking (p_0/q_0) versus the concentration of CM-chitin, CM-chitosan, and DHP-chitosan was summarized in Figure 31.15. It was found that p_0/q_0 decrease with the increasing concentration of polymer. At a concentration of 40% solution, the p_0/q_0 is lowest among various concentrations, implying that 40% is the most effective for cross-linking. Table 31.2 shows the D_g and p_0/q_0 values of chitosan derivatives irradiated at various solution concentration.

31.3.3 Characterization of the Hydrogels

Chitosan derivatives, charged or uncharged, can form three-dimensional networks under cross-linking (Wasikiewicz et al. 2006, Yoshii et al. 2003, Zhao and Mitomo 2009, Zhao et al. 2003). Some typical properties of these hydrogel materials formed from chitosan derivatives such as CM-chitosan and DHP-chitosan are selected to be introduced here.

31.3.3.1 Swelling Behavior

The capacity to swell and hold significant amounts of solvent in their network structure is one of the most important features of the hydrogels. This property makes hydrogel the perfect materials

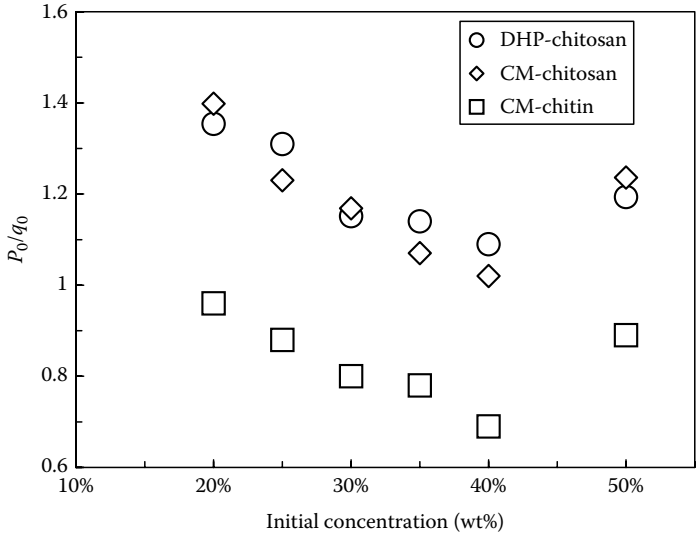


FIGURE 31.15 p_0/q_0 of CM-chitin, CM-chitosan, and DHP-chitosan gel prepared by irradiation.

TABLE 31.2
Comparison of D_g, p_0/q_0 Values of Several Chitosan Derivatives

Concentration of Polymer (%)	CM-Chitin		CM-Chitosan		DHP-Chitosan[a]	
	D_g [kGy]	p_0/q_0	D_g [kGy]	p_0/q_0	D_g [kGy]	p_0/q_0
20	9.63	0.93	47.79	1.41	36.65	1.32
30	8.37	0.80	34.09	1.10	18.38	1.16
40	8.65	0.68	32.09	0.99	9.71	1.05
50	27.8	0.74	49.68	1.20	12.68	1.18

[a] DHP-chitosan was dissolved in lactic acid solution.

as superabsorbent for solvents. The equilibrium swelling of hydrogels is a result of the balance of osmotic forces determined by the affinity to the solvent and the network elasticity. Figure 31.16 shows the equilibrium swelling degree of CM-chitosan at different doses and at various concentrations. The swelling curves indicate a typical swelling–dose relationship. The degree of swelling decreases with increasing dose in each concentration, and the gel prepared from low-concentration solution shows higher swelling ability in water than that prepared from high-concentration solution. In the perspective of practicing, therefore, chitosan derivatives hydrogels with different swelling capacities can be prepared by adjusting the dose and polymer concentration. Consequently, these novel hydrogels could meet some specific demands for the uptake and release of desired compounds or pollutants.

In the current case, DHP-chitosan is cationic polyelectrolyte, and CM-chitosan is a polyampholyte. It is well known that swelling and deswelling in response to the change of pH is a typical phenomenon of polyelectrolyte hydrogels. The typical cationic hydrogel swelling characteristics were observed in DHP-chitosan hydrogel (Zhao and Mitomo 2009). The DHP-chitosan gels swelled at acidic medium and deswelled at neutral or basic medium. The protonation of amino groups takes place at acidic solution. After gel ionization, the increased electric repulsions between positively charged amino groups cause the gel swelling. On the other hand, the typical ampholyte swelling behavior was found in the CM-chitosan hydrogels (Zhao et al. 2003). The CM-chitosan hydrogel swelled at both low pH (<3.5) and the pH range 6.0 < pH < 11.0; however, deswelling occurred

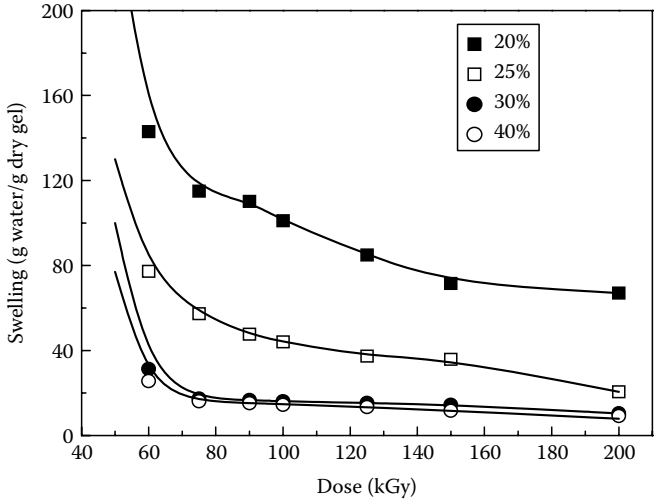


FIGURE 31.16 Swelling of hydrogels of CM-chitosan in distilled water. (From Zhao, L. et al., *Carbohydr. Polym*., 51, 169, 2003. With permission.)

in the range of pH 3.5–6.0. Due to the existence of carboxyl and amino groups, CM-chitosan gel forms a network with oppositely charged structures that could change the charged state of the ionic groups varying with pH. Considering the critical-phase transition pH, which is closed to some tissue circumstance, the pH sensitivity of these chitosan hydrogels is expected to play an important role in biomedical applications.

31.3.3.2 Mechanical Properties

The hydrogels with suitable mechanical properties are significant in the biomedical field. Only a limited literature (Wach et al. 2002, Zhao and Mitomo 2009, Zhao et al. 2003, 2008c), however, reports on the evaluation of mechanical property of polysaccharides hydrogels. In the case of radiation cross-linking, the mechanical property of hydrogels was found to be controllable by changing the cross-linking density Therefore, in the perspective of practicing, it is worthy of investigating the mechanical properties of chitosan derivatives hydrogels prepared by radiation technique.

In the case of DHP-chitosan, tensile strength and elongation at the break of hydrogels samples in relaxed state (after irradiation with the original water content) are shown in Figure 31.17. The tensile strength of both polymers increased with the absorbed dose at an early stage, after reaching the maximum, and then it decreased again. This phenomenon is owed to the increase of cross-linking density at early stage. After reaching a maximum, the higher absorbed doses led to degradation and destroyed the network structure. Elongation at the break of cross-linked polymers decreased with the increase of absorbed dose, which can be interpreted by the radiation-induced brittleness of hydrogel film. The mechanical property of radiation-induced chitosan derivatives hydrogels is dominantly controlled by the cross-linking density. The desired elasticity and flexibility of these hydrogels will be expected to meet various demands of biomedical application, such as wound dressing and tissue engineering.

31.3.3.3 Biodegradability

Biodegradability is a desirable feature of hydrogels when they are utilized in controlled drug delivery systems, superabsorbers, and other fields. In general, chitosan and their hydrophilic derivatives are biodegradable (Fukamizo 2006, Hutadilok et al. 1995, Maeda et al. 1997). Radiation-cross-linked chitosan hydrogels are environment-friendly materials since they do not produce any toxic waste products during degradation process.

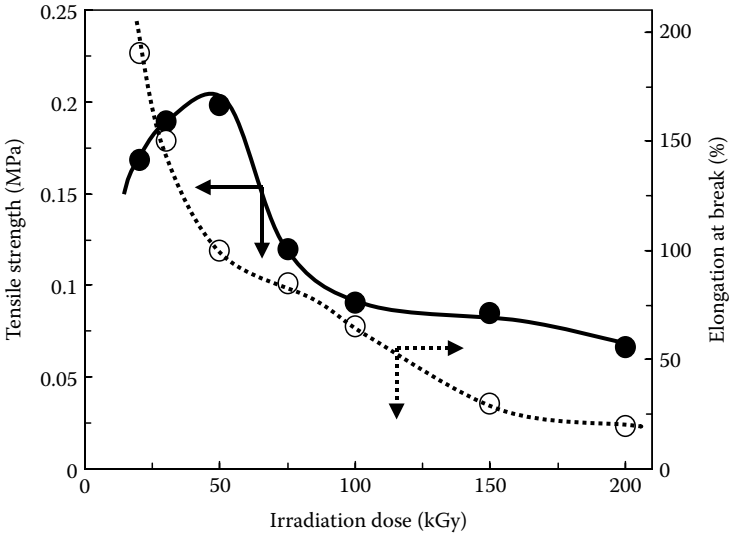


FIGURE 31.17 Tensile strength and elongation at break of DHP-chitosan hydrogels in relaxed conditions. (From Zhao, L. and Mitomo, H., *Carbohydr. Polym*., 76, 314, 2009. With permission.)

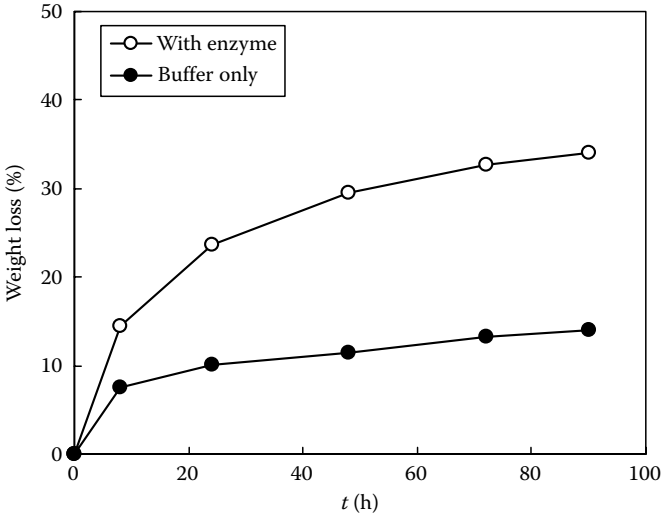


FIGURE 31.18 Biodegradation of DHP-chitosan hydrogels (prepared from 40% aqueous solution at 100 kGy) by the chitosan enzyme. (From Zhao, L. and Mitomo, H., *Carbohydr. Polym*., 76, 314, 2009. With permission.)

The cross-linked DHP-chitosan hydrogels can be degraded gradually by chitosan enzyme as shown in Figure 31.18. The hydrolytic degradation of DHP-chitosan in buffer solution is tested for comparison. The results manifested that DHP-chitosan can be efficiently degraded by enzyme even after its chains become cross-linked. The weight loss of the gel samples with and without the enzyme after 120 h incubation was 33% and 14%, respectively. The CM-chitosan hydrogels showed a similar tendency of biodegradation by chitosan enzyme.

Besides enzymatic degradation, the biodegradation behavior of chitosan derivatives hydrogels can also be evaluated by soil burial, microbial attack, and so on. The CM-chitin and CM-chitosan hydrogels before and after keeping for 10 weeks in soil is found to become smaller, revealing that

the hydrogels can be degraded in nature circumstance. A further storing of the hydrogel sample in the ground will lead to complete disintegration by bacterial activity.

31.3.3.4 Hydrodegradability with High Temperature

The thermal stability of hydrogels is very important in their application. On the one hand, the properties of hydrogels should be maintained during application, which demands good thermal stability; on the other hand, thermal degradation is preferable for the after-treatment of used gel waste. Therefore, it is necessary to investigate the thermal stability of hydrogels.

It was found that CM-chitosan hydrogels are hydrodegradable with high temperature (Wang et al. 2008). The hydrodegradation kinetics of CM-chitosan hydrogels are shown in Figure 31.19. At the temperatures of 30°C and 40°C, the hydrogels slightly degrade after heated, but keep steady in a long period. However, when the temperature rises to 60°C, the hydrogels totally hydrodegrade to sols after ca. 80 h. The thermal hydrodegradation of CM-chitosan hydrogels can be caused due to three reasons. First, the glycosidic bonds in the polysaccharide chains will cleave at high temperature. Although the CM-chitosan chains are cross-linked during radiation, forming the network structure, the cleavage of the glycosidic bonds by the acidic- or alkaline-reductive and the oxidative-reductive depolymerization mechanism (Holme et al. 2001, 2003, 2008) will occur when CM-chitosan hydrogels are surrounded by aqueous solutions with high temperature. Second, in the process of radiation-induced cross-linking of CM-chitosan, there are acylamide bonds formed. However, such bonds will hydrolyzed at high temperature (>60°C) (Kunioka and Choi 1998). Third, physical interactions in the network structures of CM-chitosan hydrogels, such as H-bond, Van der Waals attraction, electrostatic attraction, and so on, which are weaker than covalent bonds, will be destroyed at a higher temperature.

31.3.3.5 Antibacterial Activity

The hydrogels for biomedical application should be sterilized to protect them from the contamination of microorganisms (Dumitriu 1996, Rosiak and Yoshii 1999). It is well known that chitosan and its derivatives have antibacterial activity (Kumar and Majeti 2000, Liu et al. 2001, Zhao et al. 2003). Antibacterial activity can be regarded as the most valuable properties of chitosan derivatives hydrogels for biomedical application. The antibacterial activity of CM-chitosan hydrogel against *E. coli* was investigated. Figure 31.20 shows the optical density (OD) versus the culture time for the CM-chitosan hydrogel. Antibacterial activity was found to increase in the order of the gel without

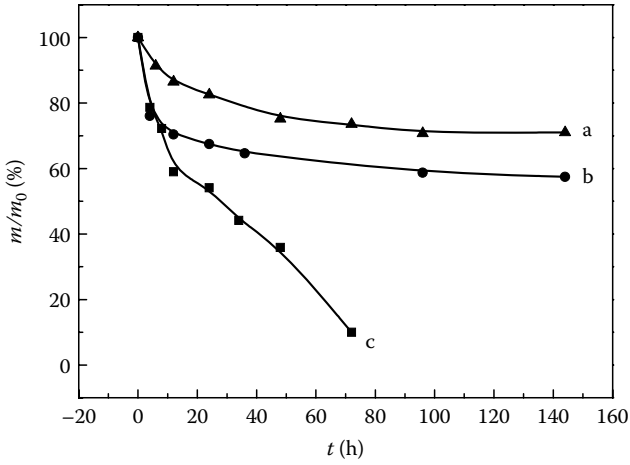


FIGURE 31.19 The hydrodegradation kinetics of CM-chitosan gels (18%, 40 kGy). (a) 30°C; (b) 40°C; and (c) 60°C.

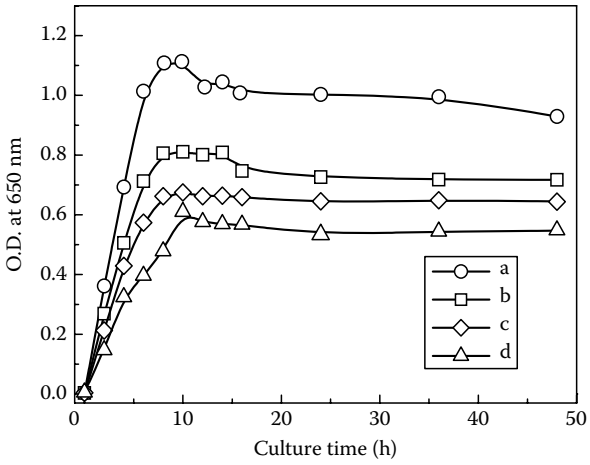


FIGURE 31.20 OD versus culture time of medium (a), CM-chitosan gel without sol part (b), CM-chitosan gel (c), and CM-chitosan raw material (d), against *E. coli* (CM-chitosan hydrogel was prepared from 40% aqueous solution at 75 kGy). (From Zhao, L. et al., *Carbohydr. Polym.*, 51, 169, 2003. With permission.)

sol part, the gel with sol part, and raw materials. No matter what the gel form, all of the samples exhibit satisfying antibacterial activity.

31.3.4 The Applications as Adsorbents

Hydrogels show a marvelous prospect for the procedure of metal-ions recovery from dilute solutions (Peter 1995, Varma et al. 2004). Recently, the adsorption of natural organic pollutants (such as humic acids) from aqueous solution using chitosan adsorbents has attracted intensive attention in environment and health fields (Ngah and Musa 1998, Wu et al. 2002, Yan and Bai 2005). Adsorbents, derived from a nature polymer, are required for environment-conscious technologies. The adsorptions of metal ions and humic acid from aqueous solutions onto radiation cross-linked chitosan derivatives were introduced here.

31.3.4.1 Adsorption of Metal Ions

Biosorption or sorption to bio-origin materials offers a technically feasible and cost-effective approach to recover metal ions from dilute solutions (Bailey et al. 1999, Volesky 2001). It was reported that chitin/chitosan and some of their derivatives are excellent adsorbents for metal ions with much higher selectivity and higher loading capacities than many commercial chelating agents (Ngah and Liang 1999). However, uncross-linked chitosans and their derivatives could not be utilized to recover metal ions due to their solubility in diluted acid solutions and poor chemical stabilization. For this reason, radiation-induced cross-linking was adopted to improve the acid-hydrolysis resistance of chitosan derivatives. The solubility test of the cross-linked CM-chitin and CM-chitosan was investigated in acidic and alkaline media, distilled water, and some organic solvents, such as acetone and ethanol. After irradiation, cross-linked samples were found to be insoluble in all of the media mentioned.

The results of various metal ions adsorption onto CM-chitin and CM-chitosan hydrogels are shown in Table 31.3 (Nagasawa et al. 2006). Each of theses metals was adsorbed separately from the solution with the initial concentration of 100 ppb at pH 4.0 in a acetic buffer. Among these metal ions, both of the hydrogels indicate a favorable adsorption of Au(III) and Cu(II) ions. Furthermore, CM-chitin hydrogels are favorable to adsorb Sc(III), Cd(II) ions, and CM-chitosan hydrogels are favorable to adsorb V(V) ions in current condition. These results demonstrated the selectivity of CM-chitin and CM-chitosan hydrogels for these metal ions. The gold (Au(III) ions) adsorption

TABLE 31.3
Metal Adsorption of Chitin and Chitosan Hydrogels

Kind of Gel	CM-Chitin		CM-Chitosan	
	Amount of Adsorption			
Kind of Metal Ion	%	μg/g	%	μg/g
Au(III)	52.6	123.6	70.8	236.2
Cu(II)	45.6	46.1	49.0	52.4
Cd(II)	34.1	40.1	2.6	25.26
Pd(II)	7.1	19.1	1.18	3.1
Pt(IV)	5.8	18.9	2.9	4.1
Sc(III)	41.73	78.15	8.42	22.3
V(V)	15.9	30.9	18.25	40.4

Source: Nagasawa, N. et al., *JAEA-Rev*., 042, 52, 2006.

onto cross-linked CM-chitosan gel is found to be 71%, which is much higher than those reported for glutaraldehyde cross-linked chitosan (58%) (Arrascue et al. 2003). The maximum uptake of Au(III) cations, based on Langmuir equation, was determined to be 37.59 mg/g for CM-chitosan and 11.86 mg/g for CM-chitin (Wasikiewicz et al. 2005a). Significant adsorption capacity together with better adsorption yield, compared to chemical cross-linking method, makes radiation cross-linked CM-chitosan an excellent gold-ion adsorbent.

Moreover, the CM-chitosan hydrogels are favorable to adsorb some heavy metal ions such as Cu(II) and Cd(II). Calculated from adsorption isotherm by Langmuir mode, the maximum uptake of Cu(II) and Cd(II) ions were determined to be 175.43 mg/g for Cu adsorption and 151.52 mg/g for Cd adsorption, respectively (Zhao et al. 2007). Nagh et al. reported the uptakes of Cu(II) ions on unsubstituted chitosan (80.7 mg/g) and chemically cross-linked chitosan (59.7 mg/g) in their article (Ngah et al. 2002). For the Cd(II) adsorption, Hsien et al. found adsorption capacity varied from 100 to 250 mg/g on a GA cross-linked chitosan (Kawamura et al. 1993). Comparing to the literature results, cross-linked CM-chitosan is superior when used as adsorbent for the removal of Cu(II) and Cd(II) ions. Therefore, CM-chitosan hydrogels are expected to have a wide-ranging application for the separation and concentration of metal ions.

31.3.4.2 Adsorption of Humic Acid

Humic acid, the major component of natural organic matters formed by humic substances, contains both hydrophobic and hydrophilic moieties in addition to many chemical functions groups such as carboxylic, phenolic, carbonyl, and hydroxyl groups connected with the aliphatic or aromatic carbons in the macromolecules (Davies and Ghabbour 1998). Humic acid predominantly carries negative charges in aqueous solution due to the presence of carboxylic and phenolic groups. Removing humic acid from water is a significant issue in water treatment. The deacetylated amino groups in chitosan and its derivatives can be protonated, and the polycationic properties of these polymers can be expected to contribute to the charged interactions with anionic substances, such as humic acid. Few reports were concerned with humic acid removal using the adsorbent based on chitin, chitosan, and their derivatives (Ngah and Musa 1998, Wu et al. 2002, Yan and Bai 2005). The adsorption behavior for humic acid using radiation cross-linked CM-chitosan was introduced here (Zhao et al. 2007, 2008a).

As shown in Figure 31.21, the capacity for humic acid adsorption is found to increase with decreasing pH, and there was hardly any adsorption of humic acid when pH exceeding 7. A sharp decrease of humic acid adsorption was observed at pH between 3.5 and 7.2. The maximum adsorption of humic acid on cross-linked CM-chitosan was found at pH 3.5. Since the zeta potentials of cross-linked CM-chitosan are negative from pH 6, the electrostatic repulsion between humic

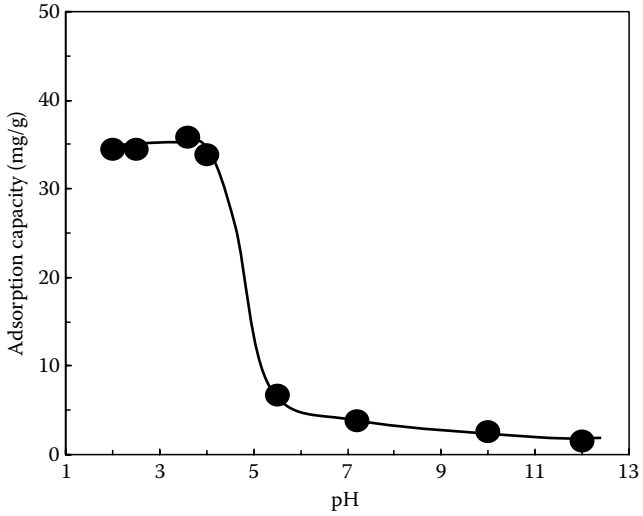


FIGURE 31.21 Effect of pH on humic acid adsorption onto cross-linked CM-chitosan (prepared from 40% aqueous solution at 100 kGy). Humic acid concentration 50 ppm, adsorption time 24 h. (From Zhao, L. et al., *Nucl. Sci. Tech*., 18, 42, 2007. With permission.)

acid molecules and the cross-linked CM-chitosan prevented humic acid from approaching the adsorbent surface, resulting in the interfere of humic acid adsorption. On the other hand, at pH below 6, the protonation of the amino groups of cross-linked CM-chitosan induced electrostatic attraction, which led to significant adsorption of the humic acid. Adsorption equilibrium studies were also carried in optimum pH condition (pH 3.5). In the current case, adsorption isothermal data were interpreted well by the Langmuir equation. The uptake of humic acid was 57.14 mg/g on cross-linked CM-chitosan at pH 3.5. It was reported that a chitosan adsorbent with the deacetylation degree of 96% get an adsorption capacity of 60 mg/g (Wu et al. 2002). Comparing their results, cross-linked CM-chitosan with 84% deacetylation degree is more efficient on the removal of humic acid. Current study clear confirms that the radiation cross-linked CM-chitosan has a potential in the applications of separation and concentration of humic acid.

31.4 CONCLUSIONS

Chitosan and its derivatives can be degraded by the scission of glycosidic bonds under irradiation. Degradation behavior was dependent upon irradiation conditions, for example, the required dose to prepare low-molecular-weight sample was lower in aqueous solution than that in solid state, and the presence of H_2O_2 improve significantly the degradation of chitosans. Pulse radiolysis and laser photolysis have been used to elucidate the radiolysis mechanism of chitosan and its derivatives. Compared to the traditional methods such as acidic hydrolysis or enzymatic treatment, radiation method prevents the usage of initiators, resulting in high-purity products. Therefore, radiation method is easy to handle and environment friendly. These radiation-degraded low-molecular-weight chitosan and its derivatives could induce antioxidant activity and antimicrobial activity, promotion of plant growth, suppression of heavy metal stress, and phytoalexins induction, etc.

On the other hand, the radiation cross-linking of chitosan derivatives has resulted in the production of biodegradable hydrogels. CM-chitin, CM-chitosan, and DHP-chitosan were cross-linked at high-concentrated aqueous solution (more than 10%, paste-like state). These hydrogels with functional groups exhibited superior water-absorption ability, satisfying biodegradability, hydrodegradability with high temperature, and biocompatibility, which is useful as the adsorbents for metal ions and organic pollutants, as well as the components of biomedical, agricultural, and cosmetic materials.

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32 Applications of Chitosan Oligosaccharide and Glucosamine in Dentistry

Yoshihiko Hayashi

CONTENTS

32.1 INTRODUCTION

Chitosan has a variety of biological activities. In dentistry and related areas, the anti-inflammatory reaction, the acceleration of the wound-healing processes in both the soft and the hard tissues, and the antibacterial effect (the bacteriostatic and/or the bactericidal actions, the differences between the Gram-positive and the Gram-negative bacteria) are mainly investigated to determine its relationship to clinical applications (Asaoka 1996, Koide 1998). This chapter focuses on the biomedical application of the chitosan oligosaccharide and the chitosan monomer (D-glucosamine). An understanding of the texture and the conformation of these biomaterials are indispensable for promoting the theoretical and practical applications. Furthermore, chitosan is also applied as the scaffold and as a carrier for molecular therapies, such as the drug and the gene delivery systems. These biomedical applications are completed through interdisciplinary collaborations.

32.2 THE MECHANISMS OF THE BIOLOGICAL ACTIVITIES

The interactions between the exogenous chitosan used in a wound and the human cells have been widely investigated (Muzzarelli 1997). Lysozyme, normally produced by macrophages, hydrolyzes the susceptible modified chitosan into oligomers, which activate the macrophages to produce nitric oxide, activated oxygen species, tumor necrotic factor-α, interferon, and interleukin (IL) 1. The activated macrophages increase their production of lysozyme, chitinase, and

N-acetyl-β-D-glucosaminidase, which catalyze the total depolymerization to monomers. The monomeric aminosugars become available to the fibroblasts, which proliferate under the action of IL-1, for incorporation into chondroitin 4- and 6-sulfate, hyaluronan, and keratan sulfate, thus guiding the ordered deposition of collagen, which is also influenced by chito-oligomers.

There are many natural organic compounds that contain nitrogen with biological activities. For example, the amino group containing nitrogen is exposed to the various degrees of deacetylation of chitin. Chitosan was shown to have numerous pharmacological actions, such as immunopotentiation, antihypertention, serum-cholesterol-lowering, and wound-healing-promoting properties (Asaoka 1996, Koide 1998). These biological effects depend on a wide range of molecular weights and the degree of deacetylation (DDA) (Hidaka et al. 1999, Singla and Chawla 2001, Ikeda et al. 2002, Fujiwara et al. 2004, Huang et al. 2004, 2005, Verheul et al. 2009). In these backgrounds, the water-soluble and the low molecular chitosans, such as the chitosan oligosaccharide and the monomer, have recently attracted the attention of researchers.

The physical property of chitosan in the cell wall/membrane is related to the electric charge. This means that cationic antibacterial agent, such as chitosan, interact with and disrupt the wall/membrane structure (Chung et al. 2004, 2005, Vishu Kumar et al. 2005, 2007, Chung and Chen 2008) (Figure 32.1). In the Gram-positive bacteria, the cell membrane is covered by a cell wall composed of 30–40 layers of peptidoglycans, which contain GlcNAc, *N*-acetyl-muramic acid, as

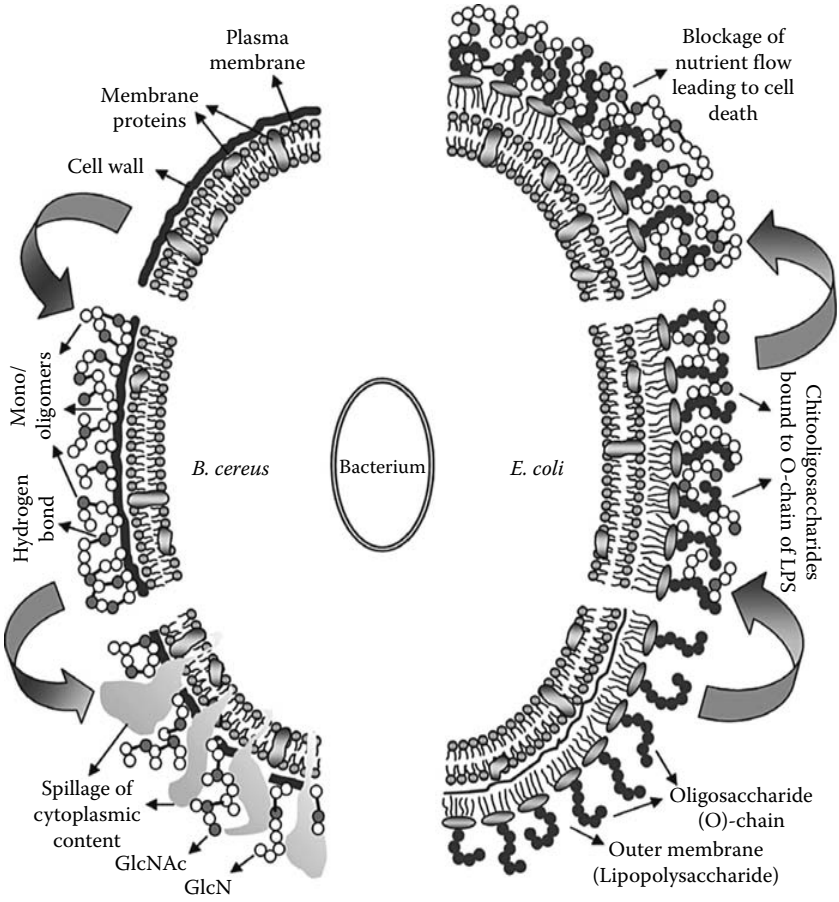


FIGURE 32.1 Mechanism of bactericidal action of chito–oligomeric–monomeric mixture towards *B. cereus* and *E. coli*: a hypothetical model. LPS, lipopolysaccharide. The large arrows indicate the sequence of bactericidal actions. (From Vishu Kumar, A.B. et al., *Biochem. J.*, 391, 167, 2005. With permission.)

well as D- and L-amino acids including isoglutamate and teichoic acid, to which the positively charged amino groups of the chito-oligomers/GlcN can bind, thus resulting in cell-wall distortion, exposure of the cell membrane to osmotic shock, and exudation of the cytoplasmic contents. The Gram-negative bacteria contain an outer membrane. The negatively charged O-specific antigenic oligosaccharide-repeating units of the *E. coli* lipopolysaccharide form an ionic-type of binding with the amino groups of the chito-oligomers, thus blocking the nutrient flow with concomitant bacterial death due to the depletion of the nutrients.

32.3 TEXTURE AND FORM

The powder form of the chitosan oligomer and the monomer is directly applied to the tissue. In the cases of cell culture or inside of the tissue, they are immediately dissolved in physiological saline or an appropriate solvent, and then added as a supplement to the culture medium or injected into the tissue. Furthermore, after the natural drying process in the culture dish, the cells or the organs are directly cultured on the sheet-like chitosan for the proliferation and the differentiation experiments. The D-glucosamine hydrochloride, as a chitosan monomer, is easily produced by total hydrolysis of chitin with hydrochloride, and it belongs to the simplest form of chitosan. The physical properties of the chitosan oligomer and the monomer are poor because of their water-soluble characteristics. The combination with other polymers or with high molecular weight materials is useful and reasonable to overcome this disadvantage. For example, the sponge-like biomaterial can be produced after mixing with polylactic acid or collagen (Tan et al. 2001, Niu et al. 2009, Zhu et al. 2009), and lyophilization.

32.4 THE BIOMEDICAL (DENTAL) APPLICATIONS

Chitosan has been widely used as an effective medicament in various fields of medicine and dentistry. Chitosan oligosaccharide and D-glusosamine are mainly applied to promote wound healing, bone regeneration, and antibacterial effect.

32.4.1 Wound Healing

Chitin/Chitosan has been widely used as an effective medicament in various medical and dental fields (Shigemasa and Minami 1996, Muzzarelli et al. 1999). In endodontics, it could be used as an anti-inflammatory root canal dressing material for the periapical lesions (Ikeda et al. 2000). Chitosan stimulates the fibroblastic cells to release the chemotactic inflammatory cytokines, especially IL-8 (Mori et al. 1997). The histological data showed that chitosan induced the migration of the polymorphonuclear leukocytes and the macrophages in the applied tissue at the early stage (Hidaka et al. 1999, Lu et al. 1999, Ueno et al. 1999). At the final stage of wound healing using chitosan, angiogenesis, the reorganization of the extracellular matrix, and the granulation tissue have been demonstrated (Okamoto et al. 1993, Peluso et al. 1994, Usmai et al. 1994, Minami et al. 1997). In the case of direct pulp capping as a biological pulp treatment, we reported that the chitosan polymer produced severe inflammation in the pulp at the early stage (Ikeda et al. 2000, Yanagiguchi et al. 2001). We used the chitosan oligomer (the mixture ranged from the dimer to the octamer) on the dental pulp tissue and observed similar initial inflammation (unpublished observations of Yanagiguchi et al.). The pulp is surrounded by the hard tissue, dentine. The inflammatory reactions are believed to cause fatal damage to the pulp tissue.

The chitosan monomer accelerates the cell proliferation and differentiation *in vitro* at a super-low concentration (Matsunaga et al. 2006). The ideal tissue regeneration in the pulp wounds occur where there is a minimal initial inflammation.

32.4.2 Bone Regeneration

The chitosan oligomer and the monomer enhanced the expression of the BMP-2 mRNA, and the ALP activity was also increased after the chitosan application in an osteoblastic cell line (Ohara et al. 2004, Matsunaga et al. 2006, Ganno et al. 2007). ALP is a marker for only the initial osteoblast differentiation in conjunction with the cell proliferation (Bellows et al. 1991, Harrison et al. 1995). The chitosan monomer might be produced by the intracellular digestion of the phagocytosed chitosan, because chitosan has an excellent bioactivity in bone healing (Broth et al. 1992, Muzzarelli et al. 1998, Hidaka et al. 1999). The chitosan oligomer can promote the BMP-2-mediated cell differentiation in conjunction with the acceleration of the cell proliferation and the ALP activity at the early phase of the osteoblast incubation (Ohara et al. 2004).

The degree of acetylation of chitosan plays a key role in the cell adhesion and proliferation. We previously reported the physiological degradation process of chitosan in the bone (Ikeda et al. 2002, 2005). The cotton-like chitosan (35%, 70%, and 100% deacetylation) was implanted into the alveolar bone cavities. The histopathological examination was carried out at 1, 3, 6, 9, and 12 months after the implantation. All the various types of chitosan were degraded with time, in conjunction with the bone regeneration. The cavity was almost completely filled with the newly formed bone tissue after 9 months most notably in the case of 100% deacetylation chitosan (Figure 32.2). These findings indicate that chitosan may promote wound healing in bone tissue as well as in soft tissue.

32.4.3 The Antibacterial Effect

In dentistry, two main oral infectious diseases are dental caries and periodontitis. Dental caries is caused by the demineralization of the dental hard tissues, such as enamel and dentine, which occurs through fermentation by indigenous bacteria (Takahashi and Nyvad 2008). Natural products have been recently investigated more thoroughly as promising agents to prevent diseases, especially plaque biofilm-related infection such as dental caries (Al-Said et al. 1986, Huwez and Al-Habbal 1986, Iauk et al. 1996, Aksoy et al. 2006). A 2% (W/V) of chitosan oligomer (a mixture of 2–8 aminosugars) (not chitosan polymer and monomer) directly and almost completely suppresses *in vitro* the growth of the typical cariogenic bacterium, *Streptococcus mutans* (GS-5 strain) (*S. mutans*), even at pH 6.5, in which the cervical tooth surface is not demineralized because the critical pH for the start of demineralization at the dentine is pH 6.4 (Fujiwara et al. 2004). This concentration and condition are thought to become a standard for clinical usage. Numerous antibacterials and antibiotics have been locally used against the typical periodontopathic bacterium, *Porphyromonas gingivalis* (*P. gingivalis*) to reduce plaque formation. The antibiotics have several adverse effects such as drug allergy and the development of antimicrobial resistant strains. This background has led to the research and development of natural organic antimicrobial agents, and several studies have investigated the interaction between chitosan and the periodontopathic bacteria (Choi et al. 2001, Ikinci et al. 2002, Sarasam et al. 2006). The conditions under which the chitosan oligomer can suppress the growth of the cariogenic bacteria without causing demineralization of the cervical tooth surface could similarly inhibit the growth of *P. gingivalis in vitro* (Table 32.1).

32.4.4 Oral Hygiene

Since 1948, Volker stated that gum chewing was an extremely common habit and reported that it removed on average, 80% of the residual oral debris (Volker 1948). In particular, the studies pertaining to the chewing of gum in elderly populations have shown an improvement in oral health and an acceptability within this age group (Markinen et al. 1996, Simons et al. 1997). Numerous antimicrobials and antibiotics, including chlolhexidine, spiramycin, and vancomycin, have been used against *S. mutans* to reduce plaque-mediated diseases, including dental caries (Gragg et al. 1997). However, in Japan since 1985, chlorhexidine has been prohibited for the use of mucous irrigation due to the occurrence of anaphylaxis. Recent studies have demonstrated that chewing

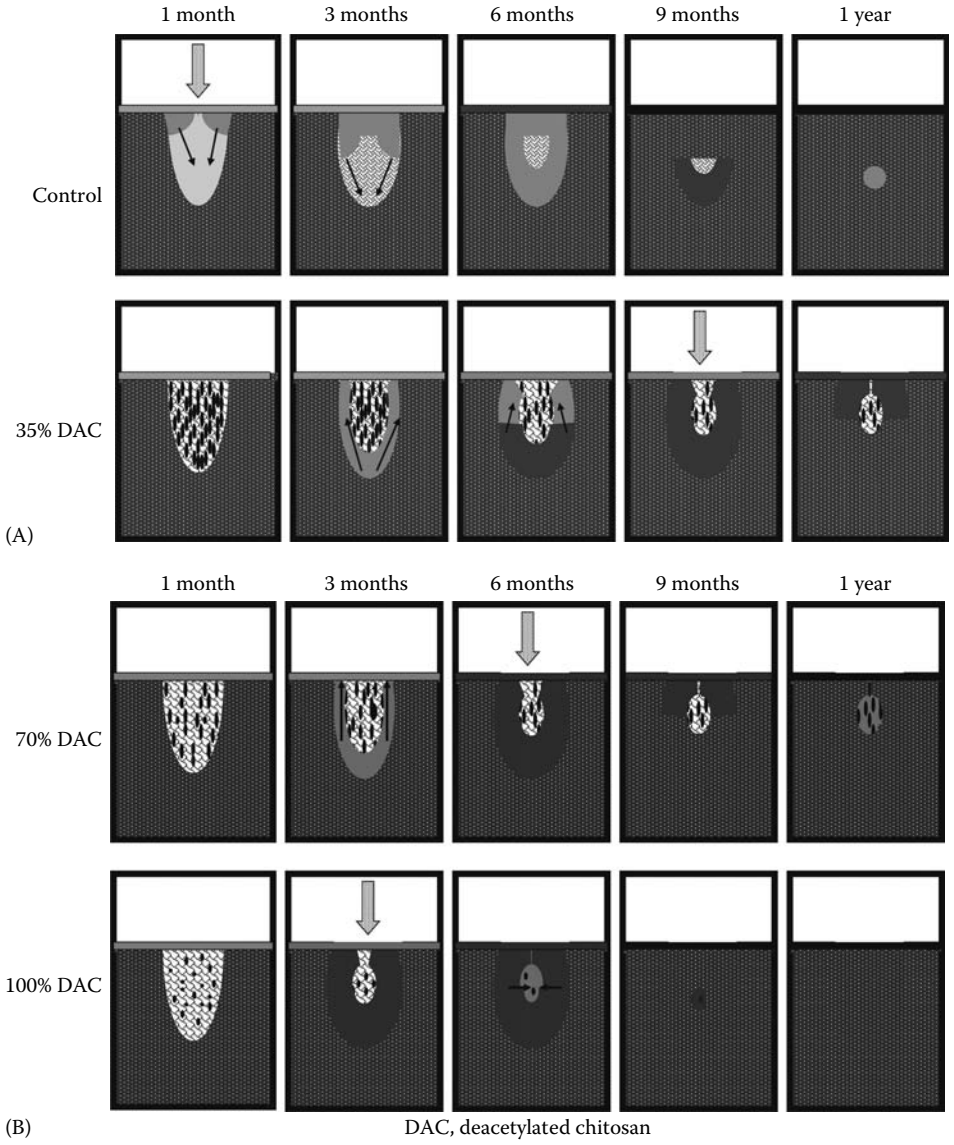


FIGURE 32.2 Wistar male rats weighing about 160 g were used for this experiment. Chitosan was kindly supplied by UNITIKA Co., Ltd. (Tokyo, Japan). The degrees of deacetylation of the specially prepared chitosan were 35%, 70%, and 100% (A, B). We refer to these as 35% deacetylated chitosan (DAC), 70% DAC, and 100% DAC. Each group was divided into five postoperative groups, representing the time periods of months, 1, 3, 6, 9, and 12. Two cylindrical cavities (about 1 mm in both diameter and depth) were prepared below the external oblique protuberance on both sides of the mandible. Three kinds of different degrees of cotton-like chitosan, weighing about 0.5 mg, were implanted in the each of the three cavities without sealing. A fourth cavity without chitosan was used as a control. Rats were conventionally perfused with fixative, and the blocks were dehydrated, and embedded in epoxy resin. Semi-thin sections (about 1 μm) were stained with toluidine blue for light microscopy. Large arrows indicate that the bony healing proceeds at the orifice of cavity. Small arrows indicate that the newly formed bone tissue is added at the inner wall of cavity. The highly deacetylated chitosan promoted the bony repair, and brought the rapid disintegration and absorption. The 100% DAC disappeared almost completely in the cavity, and the bony repair was finished at the orifice at 3 months and completely in the entire cavity at 12 months postoperatively. (From Ikeda, T. et al., *BioIndustry*, 19, 22, 2002. With permission.)

TABLE 32.1
Changes in Bacterial Growth Ratio with Increasing Concentration of Chitosan Oligomer

Concentration (W/V%)	Ratio in *P. gingivalis*	Ratio in *S. mutans* (Fujiwara et al. 2004)
0	0.980 ± 0.044	1 ± 0.061
0.01	0.899 ± 0.050	0.838 ± 0.011
0.05	0.515 ± 0.020**	0.861 ± 0.055*
0.1	0.187 ± 0.007**	0.700 ± 0.048**
0.5	0.003 ± 0.002**	0.240 ± 0.017**
1.0	0 ± 0**	0.114 ± 0.023**
2.0	0 ± 0**	0.025 ± 0.010**
4.0	0 ± 0**	0.037 ± 0.010**

Notes: Chitosan was kindly supplied by Koyo Chemical Co., Osaka, Japan. Chitosan oligomer hydrochloride is named Oligoglucosamine (DDA: 100%, a mixture of 2–8 aminosugars). A 4% (W/V) solution of chitosan oligomer was prepared by dissolving the powder-like chitosan in 0.4% (V/V) acetic acid. The completely dissolved solution was adjusted to pH 6.5 by adding sodium hydroxide solution and then filtered using a 0.22 μm filter. A similar amount of acetic acid carrier (pH 6.5) was used for the control. The antimicrobial activity of chitosan oligomer was examined against typical periodontopathic bacterium: *P. gingivalis* (ATCC No.BAA-308). The inoculum suspensions were prepared from fresh broth cultures and adjusted to obtain a concentration of 5×10^3 CFU/mL in each chitosan and control solution and then incubated at 37°C for 1 h. The mixture solution (100 μL) was inoculated onto BHI agar (BD, New Jersey, United States) medium supplemented with 0.0025% haemin and 0.0005% vitamin K, plated on plastic petri-dished and then incubated at 37°C for 24 h. After incubation, the colonies were counted to indicate the bactericidal activity, which was calculated by the growth ratio (the colony numbers in treatment/those in control). Furthermore, these ratios were calculated for the different concentrations of three types of chitosan at constant pH 6.5. All statistical requirements were met for analysis unless otherwise noted. Paired Student's *t*-test was applied to assess any significant difference in the data measured. Significance was set at a probability level of $p < 0.01$. This table shows the growth ratios in the different concentrations of chitosan oligomer at constant pH 6.5. Chitosan oligomer could strongly and significantly inhibit the growth of *P. gingivalis* at a pH value of 6.5. Chitosan oligomer completely suppressed the bacterial growth at over 0.5% (W/V).

Ratio: (Treatment vs. control) ($p < 0.05^*$, $p < 0.01^{**}$). Data (mean ± S.D.) are from triplicate samples.

FIGURE 32.3 Chitosan-containing chewing gum. The amount of chitosan oligomer added in the chewing gum is adjusted to dissolve in saliva about 2% (W/V).

the chitosan oligomer-containing gum (Figure 32.3) effectively inhibited the growth of the cariogenic bacteria (total bacteria, total *Streptococci*, mutant streptococci) in the saliva (Figure 32.4) (Hayashi et al. 2007a) and also the growth of periodontopathic bacteria (*P. gingivalis*) in saliva (Table 32.2). The chitosan oligomer-containing gum chewing has a greater antibacterial effects and it also increases salivary secretion (Hayashi et al. 2007b). These findings strongly suggest that the application of such natural materials as chitosan is useful for both oral hygiene and the quality of life of elderly individuals.

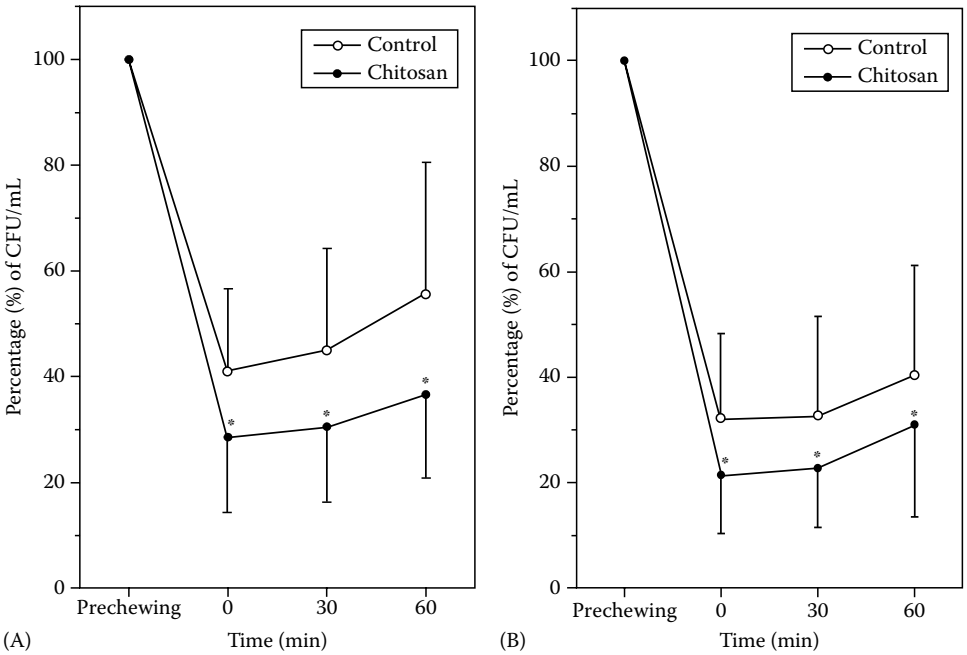
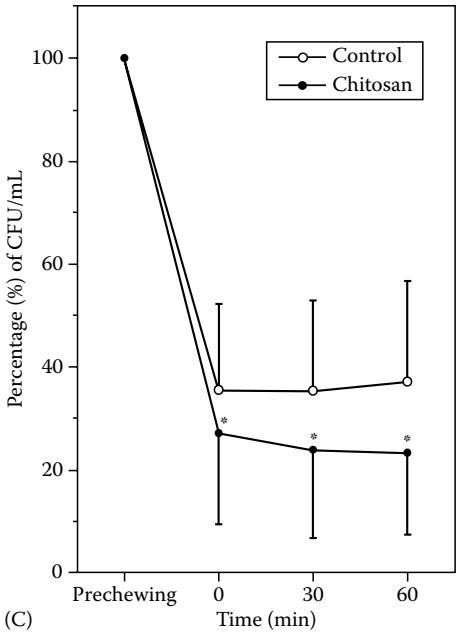


FIGURE 32.4 The *in vivo* comparative study was double-blinded and randomized. For each study, all subjects crossed over to each randomly assigned treatment, with at least one week between treatments. For 1 week before the experiment and the 1 week rest period between treatments, all subjects were instructed to use dentifrice without fluoride. The subjects brushed their teeth immediately after lunch. Two hours later, the pre-experimental saliva was collected through a 5 min wax mastication which was placed into a 50 mL centrifuge tube. The gum-chewing stage proceeded by chewing one piece of gum for 5 min followed by a rest for 5 min. This protocol was continuously repeated 8 times for 80 min at the same day. After eight pieces of gum were chewed, post-experimental saliva was collected at the following times: 0, 30, and 60 min. All saliva samples were stocked in an ice-cold container until examining the bacteria. Paraffin-stimulated saliva samples were homogenized by ultra-sonication under ice-cold water for 10 s. Serial 10-fold dilutions of the suspensions were prepared in PBS. Aliquots of the appropriate dilutions were plated in triplicate on sheep blood agar for total oral bacteria, on *Mitis-salivarius* agar for total *Streptococci*, and on *Mitis-salivarius* agar containing 0.2 U/mL bacitracin for MS. The plates were incubated anaerobically (85% nitrogen, 10% hydrogen, and 5% carbon dioxide) at 37°C for 48 h. The colony numbers were enumerated under an illuminator and the results were averaged. Colony counts obtained from pre-treatment saliva were indicated as 100% and those obtained from post-treatment saliva at three different time points were converted as percentages against the original 100%. Statistical analysis was by two-way repeated measures analysis of variance (ANOVA) to compare growth level of bacteria between both groups for 1 h after gum-chewing. The statistical difference between the groups at each sampling time was assessed using the independent *t*-test. (A) The relative change (%) of total bacteria before and after gum-chewing. Means (chitosan vs. control) followed by asterisk are statistically different ($p < 0.05$). Data are from triplicate samples. (B) The relative change (%) of total *Streptococci* before and after gum-chewing. Means (chitosan vs. control) followed by asterisk are statistically different ($p < 0.05$). Data are from triplicate samples.

(*continued*)



(C)

FIGURE 32.4 (continued) (C) The relative change (%) of mutans streptococci before and after gum-chewing. Means (chitosan vs. control) followed by asterisk are statistically different ($p < 0.05$). Data are from triplicate samples. (From Hayashi, Y. et al., *Arch. Oral Biol.*, 52, 290, 2007. With permission.)

32.5 THE SCAFFOLD AND THE CARRIER FOR MOLECULAR THERAPY

The scaffold functions to (a) provide structural integrity and to define a potential space for the engineered tissue; (b) guide the restructuring that occurs through the proliferation of the donor cells and the in-growth of the host tissue; (c) maintain distances between the parenchymal cells that permit diffusion of the gas and nutrients and, possibly, the in-growth of vasculature from the host bed; and (d) transmit the tissue-specific mechanical forces to cue the behavior of the cells within it (Marler et al. 1998).

32.5.1 The Drug Delivery System

The chitosan sponge (Figure 32.5) was developed as an osteoconductive material which induces or stimulates bone formation (Madihally and Matthew 1999). Many previous reports described the enhanced wound healing and the homeostatic effect of chitosan (Borah et al. 1992, Klokkevold et al. 1992, Chandy and Sharma 1993, Muzzarelli 1993, Muzzarelli et al. 1993, 1994, Shiraishi et al. 1993, Berscht et al. 1994). Furthermore, chitosan can regulate the release of the bioactive agents such as antibiotic and growth factors (Chandy and Sharma 1990, 1993, Muzzarelli 1993, Hirano 1996, Patel and Amiji 1996, Vasudev et al. 1997, Senel et al. 2000). The platelet-derived growth factor-BB (PDGF-BB) has been widely investigated for this drug delivery system (Park et al. 2000a). Despite the superior activity of the PDGF-BB in tissue regeneration, a rapid clearance of the PDGF-BB, due to the short half-life which makes it difficult to maintain the therapeutic concentrations from the injections, has led to the administration of extremely high doses (above 10 μg). Therefore, it is essential to develop a carrier system that maintains the PDGF-BB at the therapeutic concentration levels (1–10 ng/mL).

The sponge-like porous chitosan matrix (chitosan sponge) was used to deliver the PDGF-BB, but it also showed an initial burst effect. The burst release was probably due to the ionic repulsion

TABLE 32.2
Changes in *P. gingivalis* Concentration (%) in Saliva after Gum Chewing

	Chitosan Group			Control Group		
Treatment Period	**Pre-Experiment**	**Post-Experiment**	**Inhibitory Ratio**	**Pre-Experiment**	**Post-Experiment**	**Inhibitory Ratio**
Short	1.07	0.11	0.9	0.019	0.81	–41.63
	0.00024	0	1	0.00028	0.000047	0.83
	0.055	0.0045	0.92	0.013	0.024	–0.85
Medium	0	0	0	0	0	0
	0.0069	0.00035	0.95	0.00077	0.0047	–5.1
	0	0	0	0	0	0
	0.6	0.004	0.99	0.057	0.0005	0.99
	0.28	0.12	0.57	0.3	0.22	0.27
Long	0.09	0.051	0.43	0.18	0.31	–0.72
	1.07	0.094	0.91	0.00028	0.39	–1391.86

Notes: Data are from for triplicate samples. Ten healthy adult male subjects ranging in age from 27 to 66 years (a mean age: 49) were recruited for antibacterial activity. Approval for the studies was obtained from the Nagasaki University Graduate School of Biomedical Sciences Human Research Ethics Committee and all subjects provided their informed consent. All volunteers whose clinical diagnosis was chronic periodontitis had at least 20 natural teeth. None of the subjects were using antibiotics. Tooth brushing was completed until 2 h before saliva collection. For each study, all subjects crossed over to each assigned treatment, with at least 1 week between treatments. The xylitol-based chewing gum (pellet type, 1.5 g per piece) used for the *in situ* studies was identical for both groups except for the supplementation of chitosan in the experimental group. The amount of chitosan oligomer added in the chewing gum was adjusted to dissolve in saliva about 2% (W/V). Three outpatients were engaged for a short period treatment. The gum chewing stage involved chewing two pellets of gum for 5 min followed by a rest for 5 min. This protocol was continuously repeated three times. Five squid fishermen were engaged for a medium period treatment (four times per one night). Four courses of 5 min gum chewing on the fishing ship were carried out with the following schedule. The first time was during moving to a fishing area. The second time was after a dinner (6–7:00 PM). The third time was after a midnight meal (0–1:00 AM). The fourth time was during moving to a fishing port. Two outpatients were engaged for a long period treatment. Five times of 5 min gum chewing were carried out at 10:00, 11:30, 14:00, 15:30, and 17:00 for 1 week. The pre- and post-experimental saliva was collected through a 5 min wax mastication and then was placed into 15 mL plastic cup. Bacterial amount was quantitatively calculated by real-time PCR (Yoshida et al. 2003) and presented as the number per milliliter. For comparison of the inhibitory effect to the control group, the *P. gingivalis* amount was converted as percentage of the total bacteria at both the pre- and post-treatment of each experimental period. Under 0.00001% was interpreted as 0.0000%. The statistical difference of the inhibitory potential between groups after gum chewing was assessed using Wilcoxon rank sum test. This table shows the concentrations of *P. gingivalis* on pre- and post-experiments and the inhibitory ratio in both groups. Inhibitory ratio means (pre-experiment value – post-experimental value)/pre-experimental value. Although *P. gingivalis* was not detected in two subjects on a medium period experiment, the chitosan-containing gum chewing showed statistically significant inhibition of *P. gingivalis* growth in saliva ($p = 0.0117$) after all experimental periods. Commercial-based control gum chewing did not always suppress the bacterial growth. In fact, the data after the long period of treatment demonstrated an acceleration of bacterial growth.

between chitosan and PDGF-BB. To obtain a steady release of the PDGF-BB, the use of additional materials, such as chondroitin-4-sulfate (CS), has been proposed, based on its ionic interaction with the PDGF-BB or with chitosan (Park et al. 2000b). Since CS is negatively charged, an interaction with the positively charged chitosan or with the PDGF-BB is anticipated. The interaction between PDGF and CS induces a prolonged release of the PDGF-BB from the sponge. The release rate of the PDGF-BB could be controlled by varying the composition of CS in the sponge or by an initial loading the content of the PDGF-BB. The CS-chitosan sponge may be beneficial for enhancing the bone

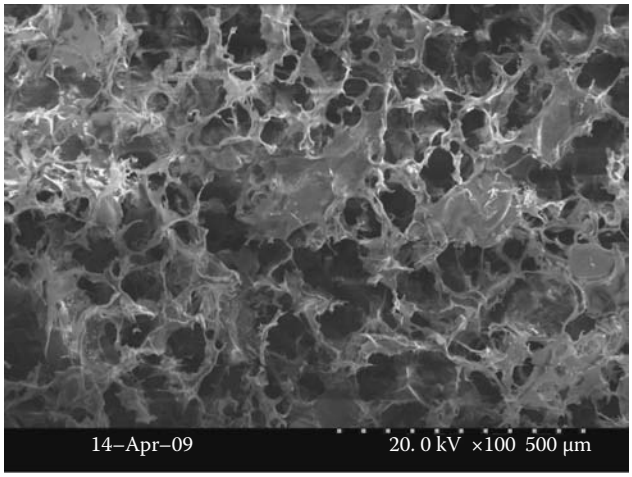


FIGURE 32.5 Chitosan polymer, FL-80 (MW: 100,000, DDA: 85%) was supplied by Koyo Chemical Co., Osaka, Japan. 2% chitosan solution in 2% acetic acid was prepared. This solution was finally neutralized to pH 7.4 using 1N NaOH. Air in solution was taken off in vacuum. The chitosan solution was freeze-dried at −80°C. The freeze-dried samples were mounted on aluminum holders and coated with carbon using a vacuum evaporator. They were examined in a Hitachi S-3500 SEM at 20 kV. The pore size in chitosan sponge was 50–200 μm (×100). The thickness of wall in sponge was almost homogenous. (Courtesy of Dr. Ikeda, T., Nagasaki University Hospital, Nagasaki, Japan.)

cell adaptation. The steady release of the PDGF-BB from the chitosan sponge can also be obtained due to the formation of the chitosan-poly (L-lactide) complex (Lee et al. 2002).

32.5.2 The Gene Delivery System

Previous studies have shown that the chitosan polyplexes exhibit low cytotoxicity and are suitable for gene delivery to the mucosal tissues, such as the intestine and the lung *in vivo* (Koping-Hoggard et al. 1998, 2001, 2003, MacLaughlin et al. 1998, Roy et al. 1999, Kumar et al. 2002, Iqbal et al. 2003). In most of these reports, the commercially available chitosans with high molecular weights (100–400 kDa) were investigated (MacLaughlin et al. 1998, Koping-Hoggard et al. 2001, 2003). These chitosan form extremely stable polyplexes with the DNA, which delayed the release of the pDNA, and the physical shape of the polyplexes was dominated by the aggregates. Other pharmaceutical drawbacks with the high-molecular-weight chitosans include their low solubility at the physiological pH and their viscosity enhancing properties at the concentrations suitable for *in vivo* gene therapy. In order to overcome these disadvantages of the high molecular chitosan for the gene delivery system, a low-molecular-weight chitosan was produced by random depolymerization, with a well-defined distribution of the oligomers (10- to 50-mers) (Koping-Hoggard et al. 2004). The polyplexes based on the chitosan oligomers dissociated more easily than those of a conventional high-molecular-weight chitosan, and effectively released the pDNA. Furthermore, 24 h after the intratracheal administration, extremely higher luciferase gene expression was also demonstrated in the mouse lung *in vivo.*

This innovative experiment demonstrates that the polyplexes of the oligomer fractions have superior physicochemical properties in comparison to the commonly used high-molecular-weight chitosan.

A new application of the chitosan monomer has tested for its ability to accelerate the efficiency of gene transfection to cells *in vitro.* In electroporation, the electric pulse is induced into the cultured cells in the dish, in which the chitosan monomer is added as a supplement to the culture medium. A high efficiency of gene transfection into osteoblastic cells was observed in comparison to the

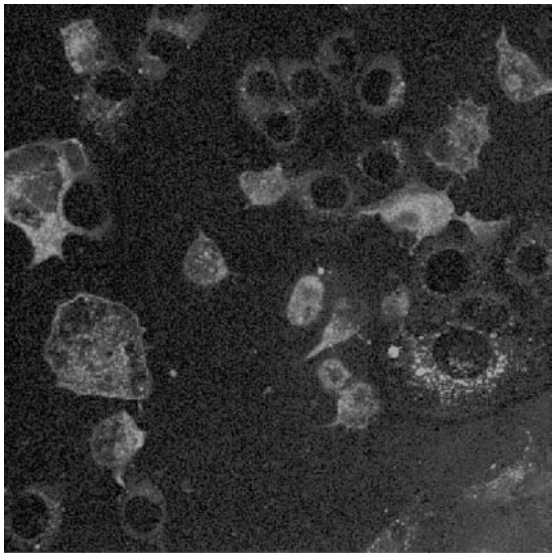


FIGURE 32.6 A 0.005% of D-glucosamine hydrochloride (Koyo Chemical Co., Osaka, Japan) was added to the buffer during electroporation of plasmid (pIRE2) containing EGFP to NOS-1 cells. Green fluorescence was detected in NOS-1 cells using a laser microscope at 1 day after treatment.

electroporation conditions only (Figure 32.6). This property may be brought by the repairing and recovery effects to the cell membrane.

32.6 CONCLUSIONS

There are many natural organic compounds containing nitrogen, which have biological activities. One of them is the amino group containing nitrogen exposed by varying degrees to deacetylation of chitin. Various biological effects depend on a wide range of molecular weights and DDA. Water-soluble and low molecular chitosans such as chitosan oligosaccharide and monomer have recently the attention of attracted researchers.

Furthermore, chitosan oligomer is not a major type of chitosan, but it has unique biological effects, such as bactericidal and bacteriostatic activities. The oligosaccharides as well as chitosan exhibited better inhibitory effects against Gram-positive in comparison to Gram-negative bacteria. Chitosan gum is the first application as a luxury good in the world. Another biological action of chitosan oligomer is that a super-low concentration of chito-oligosaccharide could potentially modulate the activity of osteoblastic cells through mRNA levels that are involved in cell proliferation and differentiation.

Glucosamine has been used for the treatment of osteoarthritis for more than 30 years. In large clinical trials, glucosamine was not effective without a combination with chondroitin sulfate in a U.S. trial, but glucosamine alone was effective in a European trial. Chitosan monomer acts as a biocompatible and stable medicament even at the initial stage of wound healing in comparison to the application of chitosan oligomer and polymer. The chitosan monomer directly affects signal transduction inside the cells *in vitro*. Therefore, glucosamine is a useful medicament for tissue engineering and tissue repair by tissue and/or cell protective activity.

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33 Applications of Chitosan and Its Derivatives in Veterinary Medicine

Sevda Şenel

CONTENTS

33.1 INTRODUCTION

Chitosan is obtained by *N*-deacetylation of chitin, which is a cross-linked polymer of *N*-acetyl-D-glucosamine and is the major structural component of exoskeletons of arthropods, such as crustaceans and insects, and fungal cell walls. Due to its favorable biological properties, such as nontoxicity, biocompatibility, and biodegradability, chitosan has attracted a lot of attention in biomedical fields. Chitosan has been favored more in these applications owing to its better solubility than that of chitin, and its free amine groups that behave as active sites in many chemical reactions. There has been a lot of interest in the application of chitosan in veterinary medicine for its bioactive properties such as wound healing, tissue regeneration, and hemostatic and antimicrobial effects, and also for its potential applications for drug and vaccine delivery in veterinary species. Chitosan is available for a wide variety of chemically modified derivatives that also find widespread utilization in such applications.

This chapter reviews applications of chitosan for its bioactive properties and in veterinary drug/vaccine delivery, and also discusses possibilities and limitations in regard to biopharmaceutical aspects. The properties of chitin and chitosan expected to enhance the therapy in veterinary medicine will be conferred and the future research directions in this field will be indicated.

33.2 DELIVERY SYSTEMS

In veterinary medicine, the development of vaccine/drug delivery systems is intended for the livestock and companion animal populations. Livestock animals are those that are raised for food or other products, or kept for use such as meat and dairy. These include not only sheep, cattle, swine, goats, and poultry but also fish and any other animals that enter the food chain. Among the companion animals (or pets), dogs, cats, and horses comprise the largest part. Other animals such as birds, reptiles, and rabbits can also be considered as companion animals. However, these species represent only a small fraction in the companion animal market.

The need for new drug delivery technology for animal health is driven by various factors such as to improve the availability of drugs, to enhance consumer convenience and compliance, to provide product differentiation, and to assure target animal and consumer safety. Although most of the companies active in the field of drug delivery are primarily interested in human medicine, the field of drug delivery technology for veterinary products provides opportunities for initiative and imaginative design in areas that are unavailable in the human pharmaceutical field. For example, there are a number of routes of administration for drugs that are inappropriate in man such as the intraruminal (Vandamme and Ellis 2004) and intramammary (Gruet et al. 2001).

Drug delivery systems are designed and developed either for conventional release or for modified release to offer the above-mentioned features. Conventional release dosage systems are preparations that show a release of the active substance(s) that is not deliberately modified, whereas in modified release dosage systems, the rate, time, and/or place of release of the active substance(s) are different from those of a conventional release dosage form administered by the same route (*European Pharmacopoeia* 2008). This deliberate modification is achieved by a special formulation design and/or manufacturing method. In the veterinary field, the definition of drug delivery systems is rather broader and includes specially designed devices to introduce the drug into the animal body. Among these are the veterinary liquid preparations for cutaneous application such as dip concentrates, pour-on preparations, spot-on preparations, sprays, teat dips, sprays, udder washes, ear tags, intramammary preparations, and intraruminal and intrauterine devices. Although many similarities between the human and animal health industries exist, there are also notable differences such as the diversity of species and breeds, the range in body size, regional differences that make the development of veterinary drug delivery systems more complicated (Ahmed and Kasraian 2002). Therefore, there are challenges and special considerations associated with veterinary drug delivery. The use of controlled (modified) release dosage forms offers numerous benefits including reducing animal stress resulting from restraint, handling, and dosing and avoiding expensive or difficult drug administration procedures. The major areas of application of controlled release technology in the veterinary field are disease prevention and control via delivery of anthelmintics, antiparasitic agents, growth promoters, nutritional agents, vitamins, and antibiotics (Haerdi-Landerer et al. 2009), especially for the food-producing animals. Although injections are common and preferred for livestock animals, oral or mucosal administration is preferred for companion animals.

Chitosan has found wide applicability in drug delivery systems due its favorable properties such as biodegradability, biocompatibility, and bioadhesivity as well as its penetration-enhancing effect (Hejazi and Amiji 2003, Berger et al. 2004, Sinha et al. 2004). The chitosan-based formulations like solutions, suspensions, tablets, gels, dispersions, films, sponges, patches, fibers, particulate (nano and micro) systems for both conventional and modified releases seem to be advantageous for oral, dermal, and especially for mucosal administration in animals.

33.2.1 Biodegradability

In controlled release systems, the duration of drug release is aimed to be extended over days up to several months. In recent years, biodegradable polymers that are biocompatible, biologically inert, and inexpensive have been widely employed for veterinary drug delivery systems owing to

the property that does not require removal from the animals at the end of the treatment period due to their degradation into physiologically occurring compounds that can be readily excreted from the body (Nair and Laurencin 2007). Currently, only a few biodegradable drug delivery systems are commercially available for veterinary use. Among the approved biodegradable polymers utilized in veterinary drug delivery are poly lactic acid, polyglycolic acid, polyurethane, and ethylene vinylacetate (Winzenburg et al. 2004). Chitosan as a biodegradable polymer has been shown to have potential use for the development of controlled release systems capable of delivering drugs within a broad range of applications (Singla and Chawla 2001, Ölmez et al. 2007). It offers several advantages over the other biodegradable polymers especially in regard to the stability of the drugs. Most of the drugs are sensitive to heat, shear forces, or organic solvents that are required during the manufacturing process of the delivery systems (especially, the particulate systems and implants) using various biodegradable polymers (Determan et al. 2006), whereas with chitosan, it is possible to avoid these undesired conditions during preparation that makes this polymer very attractive for such delivery systems without affecting the stability of the drug.

33.2.2 Bioadhesivity

Besides using biodegradable polymers, another approach to control the release of the drugs is the use of bioadhesive polymers that prolong the residence (contact) time of drug at the application site. The ability to maintain a delivery system at a particular location for an extended period of time has great appeal for both local disease treatment as well as systemic drug bioavailability (Lee et al. 2000, Gavini et al. 2002). With its excellent bioadhesive properties, chitosan is an ideal polymer for mucosal formulations.

33.2.3 Penetration-Enhancing Effect

For the drugs with low permeation across mucosa, in order to improve their absorption, the use of permeation enhancers, which increase the mucosal permeability and hence increase the availability of the drug, is required. Chitosan has been shown to exert penetration-enhancing effect across various mucosae such as nasal (Illum et al. 1994), intestinal (Schipper et al. 1997), vaginal (Sandri et al. 2004), and buccal (Şenel et al. 2000), which makes it a promising penetration-enhancing compound for the mucosal delivery of drugs without any severe effects on epithelial function and morphology.

In general, it is preferable that all or most of the characteristics required for the development of delivery systems are possessed within the same polymer system; in other words, the polymer is multifunctional. Ultimately, having all the above-mentioned properties on its own, it is evident that chitosan is more beneficial than other polymers thus exhibiting great potential as an efficient delivery system in veterinary medicine.

For veterinary drug delivery systems, additional requirements exist in regard to safety when compared with delivery systems for human. Besides the safety of the target animal, consumer safety is also important. Particularly for food-producing animals, because these animals enter the food chain, tissue residues must be addressed for both the drug and the polymer. Environmental safety should also not be affected. In view of these requirements, being a nontoxic, nonallergenic, biocompatible natural polymer, chitosan can be considered as a safe polymer for veterinary applications.

Research on chitosan-based delivery systems is undergoing rapid expansion. Recent human and animal studies have indicated that chitosan-based delivery systems are promising alternatives to conventional formulation approaches, in particular, for the mucosal delivery of biotechnology drugs, such as proteins and genes (Guang Liu and De Yao 2002, Issa et al. 2005, Bowman and Leong 2006, Lai and Lin 2009). However, the most promising results have been achieved in the area of vaccines. Section 33.3 discusses the applications of chitosan and its derivatives in veterinary vaccine delivery in detail.

33.3 VACCINE DELIVERY

Chitosan has been widely studied especially for mucosal delivery of antigens and for improving the immune response both in human and in veterinary medicines (Şenel and McClure 2004, Arca et al. 2009). It exerts great potential both as adjuvant and delivery system for vaccines due to its biodegradability, biocompatibility, and low toxicity. Furthermore, a wide choice of chitosan delivery systems such as aqueous dispersions, gels, sponges, and micro/nanoparticles has been shown to be capable of carrying antigens and other adjuvants. Compared with other delivery systems that are currently available or under investigation for antigens, chitosan-based delivery systems offer several advantages such as maintenance of stability of the antigen during preparation as well as during storage (Sayın and Şenel 2008). Therefore, highly stable formulations can be prepared with chitosan when compared with other formulations such as liposomes and ISCOMs, with which stability issue still remains as one of the major obstacles to be overcome. Moreover, chitosan itself has also been shown to exert immune-stimulating activity, although the mechanism of this action is still not clearly explained.

Chitosan derivatives have also been investigated for vaccine delivery (Gogev et al. 2004, Ghendon et al. 2008, Jiang et al. 2008, Sayın et al. 2008). Especially for antigens that are not stable in acidic pHs, water-soluble chitosan or chitosan derivatives should be used to avoid any possible inactivation of the antigen.

The recent studies on applications of chitosan and its derivatives as adjuvant/delivery systems for veterinary vaccines are summarized in Table 33.1.

Chitosan in gel and sponge form was prepared to deliver ovalbumin (Maxwell et al. 2006a) and a plant toxin, heliotrine antigen (Maxwell et al. 2006b), to sheep via rectal and nasal routes. Approximately 12 month old fine-wool Merino wethers, raised on pasture, were vaccinated by means of three vaccinations at three-weekly intervals (days 0, 24, and 43). Significant IgG1 and IgG2 responses in local mucosal (rectal) tissue and associated lymph nodes, jejunal Peyer's patch, and mesenteric lymph nodes, as well as IgA and IgE antibody levels were obtained in all tissues.

Similarly, the feasibility of inducing a protective mucosal immune response against a nonblood-feeding intestinal nematode was studied by the delivery of antigens across the mucosal epithelium (McClure 2009) in sheep. A number of antigen preparations from *Trichostrongylus colubriformis* (viable larvae, larval homogenate, and recombinant 17 kDa excretory–secretory protein) were delivered to the luminal surface of the mucosal epithelium overlying jejunal or rectal lymphoid tissue in hydroxypropylmethylcellulose cellulose or chitosan gel or sponge formulations. Delivery of *Trichostrongylus* native and recombinant antigens was reported to show significant protection of a level commensurate with that achieved by jejunal vaccination, and in mucosal (IgA and IgE) immune responses. This induction of mucosal antibody responses is consistent with the results mentioned in previous paragraph on ovalbumin and heliotrine toxin.

A mucosal vaccine delivery system for immunization against the foot and mouth disease (FMD) virus was studied using chitosan both as an adjuvant and delivery system (Çokçalışkan et al. 2007). Gel formulations were prepared using different types of chitosan, and O1 Manisa FMD virus purified antigen was incorporated into gels. The immune responses were determined in guinea pigs following intranasal administration of the formulations. The immune response was monitored for 5 weeks, during which immunization was repeated four times at weeks 0, 1, 2, and 4. Significant IgG and nasal IgA responses were obtained in presence of chitosan when compared to the control which suggests that chitosan-based delivery system is promising for inducing both systemic and local immune responses following nasal immunization against FMD.

Gel and microparticle formulations were prepared to deliver bovine herpesvirus type 1 (BoHV-1), which is one of the most prevalent pathogenic agents in the world causing serious infectious diseases most commonly, conjunctivitis, genital infections, and respiratory diseases including infectious bovine rhinotracheitis in cattle (Günbeyaz et al. 2009a,b). These formulations were shown to

TABLE 33.1
Application of Chitosan for Vaccine Delivery in Veterinary Medicine

Antigen	Delivery System	Chitosan Type	Animal Model	Administration Route	Effect	Reference
Alcelaphine herpesvirus-1	Solution (0.2% w/v in sodium acetate buffer pH 7.5)	Chitosan (Sigma, Poole, UK)	Male Friesian-Holstein cross-calves (3–5 months old)	*Priming*: Intramuscular (antigen in complete Freund's adjuvant) *Boosting*: Intranasal (antigen in chitosan)	Use of chitosan in intranasal boost led to protection of four of six animals suggesting that this approach may be worth optimizing further	Haig et al. (2008)
DNA vaccine against clumping factor A (ClfA)	Solution	Chitosan (MW: 102 kDa; DD: 70%–80%; ISM Biopolymer, Granby, Quebec, Canada)	Lactating Holstein cows	Intramuscular	Immune response was enhanced in the presence of genetic adjuvants but not with chitosan	El-Din et al. (2006)
pDNA containing major capsid protein gene of lymphocystis disease virus	Microsphere	Chitosan, MW: 1080 kDa; DD: 80%; Mingsheng Pharmaceutical Factory (Hangzhou, China)	Japanese flounder (*Paralichthys olivaceus*)	Oral	Significant immunization efficacy of microspheres loaded with pDNA and resistance to degradation in simulated gastrointestinal tract environment	Tian et al. (2008)
T. colubriformis (viable larvae, larval homogenate, and recombinant 17 kDa excretory–secretory protein) antigens	Gel sponge	Chitosan, medium MW, (Aldrich St. Louis, Missouri)	Merino ewe and wether lambs	Rectal	Induced immune response	McClure (2009)
Vibrio alginolyticus (CH003) isolated from diseased *Litopenaeus Vannamei*	Solution	Chitin (Sigma C-9752) Chitosan (Sigma Chemical Co., Saint Louis, Missouri)	White shrimp	Injection into the ventral sinus of the cephalothorax	Both chitin and chitosan increased immunity	Wang and Chen (2005)

(*continued*)

TABLE 33.1 (continued)
Application of Chitosan for Vaccine Delivery in Veterinary Medicine

Antigen	Delivery System	Chitosan Type	Animal Model	Administration Route	Effect	Reference
Bovine herpes virus (BoHV-1) glycoprotein D	Solution	Glycol chitosan (MW: 400 kDa, Sigma, Belgium) Kitomer Ultra-Pure (MW: 60 kDa; Marinard Biotech, Inc., Quebec, Canada)	Dairy calves	Intranasal	Enhanced virological protection with glycol chitosan	Gogev et al. (2004)
BoHV-1	Gel Microparticles	Chitopharm L (MW: 500–5000 kDa; DD: 70%; Chitopharm M (MW: 100–2000 kDa; DD: 70%; Chitopharm S (MW: 50–1000 kDa; DD: 70%, Cognis Protasan UP Cl 213 (MW: 150–400 kDa; DD: 75%–90%; Novamatrix)	—	—	In vitro cellular uptake and virus infectivity shown	Günbeyaz et al. (2009a,b)
Inactivated O1 Manisa foot and mouth disease virus	Gel	Chitopharm L Chitopharm M Protasan UP Cl 213	Guinea pigs	Intranasal	Induced significant IgG and nasal IgA responses	Çokçalışkan et al. (2007)
Ovalbumin	Gel sponge	Chitosan (medium MW, Sigma, New York)	Sheep	Rectal	Stimulated a combination of local and jejunal mucosal immune responses	Maxwell et al. (2006a)

Heliotrine antigen	Gel sponge	Chitosan (medium MW, Sigma)	Sheep	Rectal	Stimulated a combination of local and jejunal mucosal immune responses	Maxwell et al. (2006b)
Streptococcus equi antigens	Microspheres	Chitosan [low MW (150 kDa); medium MW (400 kDa); high MW (600 kDa); Fluka]	Female BALB/c mice	Subcutaneous	Induced humoral and cellular immune response	Florindo et al. (2008)
Pig interleukin-2 gene	Nanoparticles	Chitosan glutamate (Chengdu Organic Chemicals Co. Ltd., CAS, 95% deacylated, MW = 150 kDa)	3 week old female Kunming mice	Intramuscular	Significantly increased immunoglobulins, specific antibody, and interleukins with chitosan nanoparticles	Xie et al. (2007)
ClfA	Solution	Chitosan	Lactating cattle	Intramammary	Use of cationic transporter reduced the interanimal variability	Talbot and Lacasse (2005)
Egg yolk immunoglobulin	Chitosan-alginate microcapsules	Chitosan, (DD: 90%; Dalian Xindie Chitin Co., Ltd., Dalian, China)	K88 antibody free Belgian Landrace pigs (40-day-old)	Oral Challenged with K88 + Enterotoxigenic *Escherichia coli* (ETEC)	Successful protection of IgY antibodies from gastric inactivation leading to enhanced protection against ETEC-induced diarrhea and weight loss	Li et al. (2009)

be potential delivery systems for antigen delivery with properties of having suitable particle size to be taken up by the cells and maintaining the antigen integrity.

The ability of two soluble chitosan formulations (chitosan and glycol chitosan) to improve the immunogenicity of an intranasally administered replication-defective human adenovirus type 5 expressing BoHV-1 glycoprotein D-based vaccine was investigated in cattle (Vila et al. 2004). It was reported that soluble formulation of glycol chitosan has promising potential use as an intranasal adjuvant for recombinant viral vector vaccines in cattle.

Streptococcus equi subsp. *equi*, which is the causative agent of Strangles, was adsorbed onto poly-ε-caprolactone microspheres with chitosan-modified surfaces (Florindo et al. 2008). After subcutaneous immunization of mice, the formulations induced higher lymphokines levels than did the free antigen, in accordance with cellular and humoral immune responses, successfully activating the paths leading to Th1 and Th2 cells. More pronounced responses were observed with chitosan-surface-modified PCL microspheres than those of the unmodified formulations.

Oral DNA-based immunotherapy which is a new treatment option for fish immunization in intensive culture to prevent from lymphocystis disease has been reported to have some limitations. Due to the existence of the nucleases and severe gastrointestinal conditions, DNA-based vaccines can be hydrolyzed or denatured when given orally. Chitosan microspheres (<10 μm) that contained about twice the amount of pDNA that is generally given to fish by injection route were shown to result in significant pDNA expression in fish after oral administration (Tian et al. 2008) In comparison to immunization via injection with naked pDNA, oral administration of chitosan microspheres loaded with pDNA vaccine were reported to be more stable and last longer for the immunization of fish.

To stimulate immunity in the oro-nasal-pharyngeal region of cattle to protect them from alcelaphine herpesvirus-1-induced malignant catarrhal fever (MCF), attenuated C500 strain alcelaphine herpesvirus-1 was used either along with Freund's adjuvant administered intramuscularly in the upper neck region to immunize cattle or with chitosan as adjuvant administered intranasally (Haig et al. 2008). Protection was associated with high levels of neutralizing antibodies in nasal secretions. Some protected animals showed transient low levels of viral DNA in blood samples and in one lymph node sample after challenge, whereas viral DNA was detected in the blood and in lymph node samples of all animals with MCF. This was reported to be the most promising immunization strategy to date for the control of MCF.

In order to explore the safe and effective adjuvant for the promotion of immunity of animals against *Pasteurella* infection, Tibet pig interleukin-2 (IL-2) cDNA was shuffled with other IL-2 cDNAs from human, yak, and mouse, and the effect of shuffled IL-2 (IL-2S) gene in vivo was investigated on immunity of mice to *Pasteurella multocida*. The IL-2S protein was found to remarkably promote the proliferation of pig lymphoblasts than the native pig IL-2 protein. Then the IL-2S gene was cloned into VR1020 eukaryotic plasmid (VRIL2S) and enwrapped with chitosan nanoparticles (CNP-VRIL2S). Kunming mice were muscularly inoculated, respectively, with the CNP-VRIL2, CNP-VRIL2S, and CNP-VR1020 along with *P. multocida* vaccine, and orally challenged with virulent *P. multocida* on 28 days postvaccination. The immunoglobulins, specific antibodies, and ILs were observed to increase significantly after vaccination and challenge in CNP-VRIL2S group when compared to the control, and 9 of 10 immunized mice were reported to survive challenge. VRIL2S entrapped with CNP was suggested as a novel safe and effective adjuvant to boost the specific immunity and resistance of animal against infectious pathogen, which could facilitate the development of highly promising powerful adjuvant (Xie et al. 2007).

Clumping factor A (ClfA) was used as the vaccinating gene in lactating cattle (Talbot and Lacasse 2005). The GM-CSF gene was used as a stimulatory cytokine, chitosan as a transporter, and CTLA-4 as a targeting sequence. The immunizing efficiency of the basic ClfA plasmid was quite high, and none of the treatments were significantly superior to the naked plasmid alone. The use of the cationic transporters in a later experiment appeared to reduce the interanimal variability.

33.4 BIOLOGICAL PROPERTIES OF CHITOSAN EXPLOITED IN VETERINARY MEDICINE

The major application of chitosan and its derivatives in veterinary medicine is based on their biological activities such as wound healing, antimicrobial, hemostatic, and tissue regeneration, which meet the need to heal animal wounds quickly and effectively, especially those that are difficult to heal such as scalds, injury wounds, and wounds generated as a result of various diseases. In the following sections, these activities are described briefly and their applications in animals especially in wound management are reviewed.

33.4.1 Hemostatic

Chitosan can be used in medical and surgical procedures by its direct application to a bleeding surface using the various physical forms such as powder, solution, film, hydrogel, and filament composite (Whang et al. 2005). Each has its own advantages in regard to handling, absorption, hemostasis, and bioadhesion, and their clinical usefulness would depend on the application techniques utilized by individual surgeons for particular wound types. The chemical properties of chitosan such as molecular weight, degree of ionization, degree of deacetylation, and degree of crystallinity would also influence the hemostasis.

33.4.2 Wound-Healing Activity

Chitin, chitosan, and their derivatives have been applied for wound treatment in the veterinary field since 1988, and one of the first cases of chitosan application was reported on a case of canker in a draft horse (Minami et al. 1991). They have also summarized clinical cases using chitin and chitosan to large animals (cows and horses) (Minami et al. 1992), small animals (dogs and cats) (Okamoto et al. 1992), and zoo animals (mammals, reptiles, and birds) (Fukumoto et al. 1995) using the chitin products (Chitipack S and Chitipack P), and a chitosan product (Chitopack C), which are commercialized in Japan. In comparison with conventional therapy with irrigation and antibiotic administration to a wound, new treatments with chitin and chitosan products permitted a substantial decrease in treatment frequency with minimal scar formation.

Chitosan enhances the functions of inflammatory cells such as polymorphonuclear leukocytes (phagocytosis, production of osteopontin, and leukotriene B4), macrophages (phagocytosis, production of IL-1, transforming growth factor (TGF)-β1, and platelet-derived growth factor), and fibroblasts (production of IL-8). As a result, chitosan promotes granulation and organization; therefore, chitosan is beneficial for the large open wounds of animals (Ueno et al. 2001).

There are still many studies carried out by different groups using chitin, chitosan, and their derivatives to accelerate wound healing in clinical veterinary cases. Table 33.2 summarizes the recent studies on application of chitosan in wound management. In most of these studies, the chitin and chitosan have been used as filaments, powders, granules, sponges, or as a composite with cotton or polyester. Many remedies using chitin and chitosan for treatment of wounds have already been marketed that are used both in human and veterinary medicines (Table 33.3).

The effect of chitin on superficial wound repair as well as on healing of sheep flexor tendons was investigated using a nonwoven polyester fiber (Okamoto et al. 1997). Chitin was shown to have a beneficial effect on collagen differentiation around nonwoven fabric (NWF) tendon implants.

Tissue reactions induced by polyester NWF (1 × 1 cm^2, 0.6 mm thick) impregnated with chitin or chitosan suspension ranging from 0.1 to 50 mg/mL (chitin-NWF, chitosan-NWF) and NWF impregnated with phosphate-buffered solution (control) were investigated in rats following subcutaneous implant (Kojima et al. 2001). Evaluations were based on macroscopic and microscopic observations identifying glycosaminoglycan, proteoglycan, and collagen. Suitable levels of chitosan for organization of implants in rats were found to be 1–10 and 0.1–1 mg/mL, respectively.

TABLE 33.2
Applications of Chitosan for Wound Management in Veterinary Medicine

System	Chitosan Type	Other Excipient(s)	Animal Model	Administration Route	Effect	Reference
Dressing	Chitosan	Reinforced with bioabsorbable polymer film, TephaFLEX	Crossbred adult domestic swine	Laparoscopic	Controlled severe inferior vena cava hemorrhage	Xie et al. (2009)
Composite hydrogels	Chitosan flakes (MW: 400 kDa; Fluka)	Collagen-chitosan composite stabilized by either a simple carbodiimide cross-linker or a hybrid cross-linking system	Yucatan pigs	Corneal implant (for 12 months)	Seamless host-graft integration allowed with successful regeneration of host corneal epithelium, stroma, and nerves	Rafat et al. (2008)
Bi-layer physical hydrogel Viscous solution	Chitosan (MW: 540,000 g/mol; DD: 97.1%; Mahtani Chitosan)	—	Female mini pigs	Dermal (on dorsal burns processed)	Appropriate response induced for the reconstruction of the skin after a third-degree burn injury, on a limited area	Boucard et al. (2007)
Dressing	Cotton fiber-type chitosan (DD: 82%; Chitopack C™, Eisai Co., Ltd., Tokyo, Japan)	—	Neutered male beagles	Dermal (on irradiated wound model with full thickness-skin loss)	Substantially improved neovascularization and granulation bed formation; enhanced wound-healing process; upregulated PDGF, TGF-β1 and VEGF mRNA expression	Ueno et al. (2007)

In-situ forming gel	Ultrapure chitosan (MW: 260.5 kDa; DD: 76.3%–82.6%, Bio Syntech, Laval, Quebec, Canada)	Covalent modification of chitosan-glycerol phosphate solutions by hydroxyethyl cellulose surface-treated with glyoxal	Sheep	Implant in bone defect	Hydrogel retained less than 1 month, revealing limitations of large animal cartilage repair models, suitable for delivery of cells or agents that exert local, short-term therapeutic effects on initial cartilage repair processes	Hoemann et al. (2007)
Adhesive strip	Chitosan (DD: ≥85%) (Sigma, St. Louis, Missouri)	Genipine (cross-linker)	Sheep Rat	Implanted in small intestine Thermal damage induced by the laser irradiation of strip applied on sciatic nerve	Adhesion to the intestine serosa Enhanced adhesion by the laser at a power level of 120 mW	Lauto et al. (2005)
Film	Chitosan-H (Dainishiseika Colour and Chemicals MGF Co. Ltd., Tokyo, Japan)	Taurine (for antioxidant activity)	Female beagle dogs	Implantable topical hemostatic dressing	Accelerating effect of chitosan on wound healing at early periods enhanced in presence of taurine	Özmeriç et al. (2000)
Film	Chitosan (Practical Grade, DD: 85%; Sigma–Aldrich, St. Louis, Missouri)		Enucleated pig eyes	Sclera wound with and without the application of near infrared laser irradiation using a robotic manipulator	Both lasered and non- lasered film resulted in watertight closures immediately and after 24 h	Garcia et al. (2009)
Adhesive complex	UV-curable chitosan derivative	Synthetic resin	Cat (3-month-old)	On sutured skin to prevent postoperative pneumothorax	Showing excellent sealing effect, peeling off spontaneously from the skin after healing	Renbutsu et al. (2007)

TABLE 33.3
Some Commercially Available Chitin/Chitosan Based Dressings Used Both in Human and Animals for Wound Management

Product	Company
Beschitin® (chitin)	Unitika (Nagoya, Japan)
Chitopack-C® (chitosan)	Eisai (Tokyo, Japan)
Chitipack-S® (chitin)	
Chitipack-P®	
Clo-Sur^{Plus} PAD (poly-D-glucosamine and poly-*N*-acetylglucosamine)	ScionCardio-Vascular, Inc. (Miami, Florida)
Chitoseal® (chitosan)	Abbott (Abbott Park, Illinois)
Syvek Patch® (poly-*N*-acetylglucosamine)	Marine Polymer Technologies
HemCon® (chitosan acetate) ChitoFlex®	Hemcon (Portland, Oregon)
Celox™ (chitosan)	SAM Medical (Newport, Oregon), MedTrade Products Ltd (Crewe, Cheshire United Kingdom)

The application of chitosan hydrogels has been demonstrated to effectively interact with and protect the wound, ensuring a good, moist healing environment. An insoluble flexible hydrogel-like soft rubber was prepared following application of ultraviolet light irradiation to a photocrosslinkable chitosan aqueous solution (Ishihara et al. 2002). The resulting gel was evaluated as a dressing for wound occlusion and an accelerator in the healing process, using a mouse model with full-thickness skin incisions. Application of the gel onto an open wound induced significant wound contraction and accelerated wound closure and healing. Furthermore, in cell culture studies, it was shown that the immobilized chitosan hydrogel in the presence of fetal bovine serum had a chemoattractive influence on dermal fibroblasts and stimulated cell growth.

Photocrosslinkable chitosan molecules that are easy to apply onto various kinds of wounds and produce insoluble hydrogel by a short ultraviolet light irradiation were shown to cover and protect various wounds and accelerate healing. In addition, they were reported to effectively seal air leakage by incision on the lung and bleeding from the artery as a biological adhesive. Thus, the photocrosslinkable chitosan hydrogel has been suggested as promising dressing for wound occlusion and as a tissue adhesive, especially suitable in situations requiring an urgent hemostasis in disaster medicine (Ishihara et al. 2001, Ishihara 2002).

In order to investigate the effect of chitosan on radiation-impaired wounds that were characterized by fibroblast and endothelial cell injury, cotton fiber-type chitosan (degree of acetylation, 18%; Chitopack C, Eisai Co., Ltd., Tokyo, Japan) was applied on an x-ray-irradiated skin wound in dogs. Chitosan was shown to substantially improve neovascularization and granulation bed formation and also enhance the wound-healing process. Furthermore platelet-derived growth factor (PDGF), TGF-β1, and vascular endothelial growth factor (VEGF) mRNA expression were demonstrated to be upregulated by topical chitosan application, even under irradiated conditions (Ueno et al. 2007).

Recently, a novel photosensitive chitosan derivative, UV-curable chitosan (UVCC-7-10) for surgical adhesive was developed (Renbutsu et al. 2007). The cured gel was reported to show transparency and had good adhesive potential and good biocompatibility elasticity to a skin. The UVCC was applied to a diaphragmatic hernia in a kitten. Surgical incisions were completely sealed by UVCC after removal of air from the thoracic pleural cavity and the kitten was reported to recover normal breathing.

The effect of chemical properties of chitosan and chitin on wound healing was studied on a linear incisional wound model in rats (Minagawa et al. 2007). Wound break strength of the chitosan group (D-glucosamine, chito-oligosaccharide, chitosan) was found to be higher than that of the chitin group (*N*-acetyl-D-glucosamine, chito-oligosaccharide, chitin). Collagenase activity was also

reported to be higher in the chitosan group than that in the chitin group. No significant change was observed between the concentration of the sample and the break strength and collagenase activity in all samples. Stronger break strength and more activated fibroblasts were observed with the increased degree of deacetylation.

A composition of a selected chitosan form and its derivative, such as microcrystalline chitosan or a gel from chitosan salt with poly-*N*-vinylopyrolidone, was used to manufacture new dressings for veterinary use to protect injured animal skin successfully (Wiśniewska-Wrona et al. 2002). Strong bacteriostatic/bactericidal activity and improved mechanical properties were reported for these dressings.

Formulations using chitin nanofibrils, chitosan glycolate, and chlorhexidine were developed in spray, gel, and gauze forms, the latter including nonwoven dibutyryl chitin as a biocompatible support (Muzzarelli et al. 2007). The products were tested in murine wound models, with phytostimuline-medicated wounds as controls, in two series of experiments, one of which included a concomitant laser treatment capable to activate cells. Better epithelial differentiation and keratinization, and reorganization of the basal lamina were reported to be induced with the gauze. A single dressing could be kept on site for at least 4 days, and the healing was reported to take place within periods of time similar to those reported for traditional dressings under comparable conditions; in no case, secondary infection was developed. The spray was found to be most effective in healing superficial lesions, thus was suggested to be used as a first-aid tool on bleeding abrasions; the gel was more effective in repairing shallow lesions as well as an aesthetic factor, while the gauze was effective slow-healing dermoepidermal wound. The nanofibrillar chitin–chitosan glycolate composites were reported to exert control over various biochemical and physiological processes involved in wound healing, besides hemostasis.

33.4.3 Antimicrobial Activity

In addition to the requirements for wound healing, it is important to control any infection of a wound under dressing. Infectious organisms preferentially target wounds beneath the dressing materials and elicit serious infections that frequently require removal of the wound dressing. For these reasons, the treatment of wounds requires the suppression of bacterial growth.

Chitosan itself possesses antimicrobial activity against many fungi, bacteria, and viruses (Rabea et al. 2003). The antimicrobial action is influenced by intrinsic factors such as the type of chitosan, the degree of chitosan polymerization, the host, the natural nutrient constituency, the chemical or nutrient composition of the substrates or both, and the environmental conditions (e.g., substrate water activity or moisture or both). The antimicrobial activity of chitosan is affected by pH (pH 4.5–5.9 range tested), with greater activity being found at the lower pH values (Vinsova and Vavrikova 2008). Due to the positive charge on the C-2 of the glucosamine monomer below pH 6, chitosan is more soluble and has been reported to have a better antimicrobial activity than chitin (Rabea et al. 2003). Recently, a novel water-soluble chitin derivative, aminoethyl chitin was shown to exert antimicrobial activity (Je and Kim 2006).

The exact mechanism of the antimicrobial action of chitin, chitosan, and their derivatives is still unknown, but different mechanisms have been proposed. Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents (Helander et al. 2001, Liu et al. 2004). Chitosan also acts as a chelating agent that selectively binds trace metals and thereby inhibits the production of toxins and microbial growth. It also activates several defense processes in the host tissue, acts as a water-binding agent, and inhibits various enzymes. Binding of chitosan with DNA and inhibition of mRNA synthesis occur through chitosan penetration toward the nuclei of the microorganisms and interference with the synthesis of mRNA and proteins (Rabea et al. 2003).

Antifungal activity was reported to decrease with declining molecular mass (chitosan oligosaccharide and *N*-acetyl-D-glucosamine) and increasing masking of the protonated amino groups

with functional groups (carboxymethyl chitosan). A concentration-dependent antifungal activity of low- and high-molecular-weight chitosan hydrochloride against *Candida albicans*, *C. krusei*, and *C. glabrata* in acid medium was shown using nephelometry. These findings suggested that the polycationic character of chitosan was crucial (Seyfarth et al. 2008).

Antiviral activity of chitosan was reported to depend on the average degree of polymerization, the degree of *N*-deacetylation, the positive charge value, and the character of the chemical modifications of the molecule (Liu and De Yao 2002).

33.4.4 Tissue Engineering

Recently, functional biomaterial research has been directed toward the development of improved scaffolds and new drug delivery systems for regenerative medicine (Shi et al. 2006). In this regard, increasing attention has been given to chitosan and its derivatives that are capable of being prepared in porous forms to offer a channel for the migration of host cells into the matrix-permitting growth into complete tissue analogs and are biodegradable (through enzymatic degradation in the presence of lysozyme) once they have served their function in vivo (Khor and Lim 2003).

Chitosan and its derivatives are characterized by excellent biostimulating properties that facilitate reconstruction and vascularization of damaged tissues, and also compensate the shortcomings of cell components, which are conductive for small-scar forming. This useful feature is a consequence of several processes and phenomena such as biodegradation, influence on living cell membranes, natural conformability with living cells, and lack of toxicity. The reader is referred to the review article by Muzzarelli (2009) that extensively reviews chitins and chitosans for the repair of wounded skin, nerve, cartilage, and bone.

33.5 DIVERSE APPLICATIONS

A chitosan-based nutritional supplement (Epakitin™, Vétoquinol) that is claimed to bind phosphate and reduce urea levels is commercially available for use in chronic kidney disease in dogs and cats (Vétoquinol Web site 2009). There are commercially available dietary supplements containing chitosan that can be directly sprayed on the food for the dogs (Exotic Dogs Web site 2009). Food applications of chitin and chitosan are currently limited.

Various body care chitosan-based products such as shampoo, earcleaner, conditioner, and sprays for companion animals are also available on the Kodiak Pet Products Web site (2009).

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Part VI

Industrial Applications of Chitin and Chitosan Derivatives

34 Separation Membranes from Chitin and Chitosan Derivatives

Tadashi Uragami

CONTENTS

34.1 INTRODUCTION

Chitin is a polysaccharide that is biologically produced. In general, it is easy to obtain a large quantity of chitin from crab and shrimp shells that are considered as food waste. Chitosan is one of the few basic polysaccharides that is easily obtained by hydrolyzing chitin. Chitin and chitosan can be effectively utilized as separation membranes. This effective application of chitin and chitosan could consume a very large amount of these waste materials. When natural resource conservation, energy saving, and environmental preservation are considered, the use of chitin and chitosan also has large social advantages.

Chitin and chitosan have high organic solvent resistance, which is advantageous for separation membranes used with organic solvents, where chemical resistance is typical. Specifically, chitin is highly acid resistant and chitosan is highly alkaline resistant. These characteristics make it possible for chitin and chitosan to be used as separation membranes for a variety of uses in response to specific requirements.

Applications for separation membranes are many and diverse; separation membranes must also function in a variety of conditions. For example, when hydrophilic or hydrophobic membrane materials are desired, they can be easily synthesized because chitin and chitosan have many reactive functional groups, such as hydroxyl and amino groups. These reactive groups are useful for the introduction of functional groups and cross-linkers, and consequently function to strengthen and improve separation membranes. Since chitin and chitosan are polysaccharides derived from

biomass, they are safe to use; in particular, chitin has very low toxicity and can be used for degradation processes in living organisms. These advantages can also be applied to the development of separation membranes for medical treatment and medicine (Uragami et al. 2001; Uragami and Tokura 2006).

Chitin and chitosan have various characteristics that are not found in other natural polymers and biopolymers. However, for a long time, chitin and chitosan were unused bioresources. In the investigation of the importance of natural polymers, chitin and chitosan, as well as cellulose, are being considered functional polysaccharides, and are actively being studied and applied in various fields, such as medical treatment, medicine, food, chemical industries, fibers, and others (Uragami et al. 2001; Uragami and Tokura 2006).

In this chapter, we deal with the fundamentals and the characteristics of the derivative membranes of chitin and chitosan. These derivative membranes are expected to be fundamentally functional material that can support the development of scientific technology in the future.

34.2 SEPARATION MEMBRANES DERIVED FROM CHITIN AND CHITOSAN

34.2.1 Gas Permeation Membranes

In gas permeation, the difference in the partial pressure of gases on both sides of the membrane becomes a driving force, and gas separations through membranes are due to the difference in the solubility of gases into the membrane and their diffusivity in the membrane, as shown in Figure 34.1.

The permeabilities of oxygen and carbon dioxide of chitosan membranes prepared from an aqueous solution of acetic acid by the dry and the wet methods were studied (Sakurai et al. 1983). The permeabilities of gases were remarkably influenced by the membrane preparation method, and were significantly dependent on the fine structure of the membrane. The permeability and the sorption amount of water vapor were also affected by the kind of acid used as a casting solvent for chitosan, and the importance of the resulting membrane structure was suggested (Sakurai et al. 1984). The elongation and the CO_2 and the water vapor permeability of chitosan/poly(vinyl alcohol) (PVA) blend membranes increased with an increase in the plasticizer content (Arvanitoyannis et al. 1997). However, high plasticizer contents caused a substantial decrease in both the tensile strength and the modulus. The steady-state permeation rates for CO_2 and N_2 in dry and wet (swollen with water vapor) chitosan membranes were measured by a variable volume method. The sorption equilibrium for N_2 obeyed Henry's law, whereas that for CO_2 was described apparently by a dual-mode sorption model. This non-linear sorption equilibrium for CO_2 could be interpreted by the interaction of sorbed CO_2 with the chitosan matrix expressed as a reversible reaction. The logarithm of the mean permeability

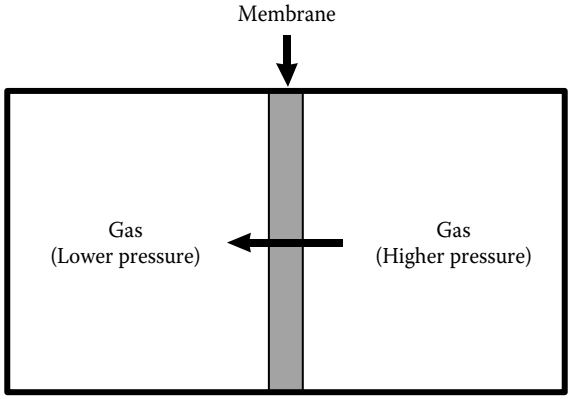


FIGURE 34.1 Principle of gas permeation through the membrane.

coefficient for CO_2 in the dry chitosan membrane increased linearly with upstream gas pressure. The permeabilities for CO_2 and N_2 in the wet chitosan membrane increased 15–17 times and 11–15 times, respectively, as compared to those in the dry membrane (Bae et al. 1998). Films of chitosan glutamate and sodium alginate were obtained by a casting/solvent evaporation method, and cross-linked with their counter ions, tripolyphosphate and calcium chloride, respectively (Remunanlopez and Bodmeier 1997). The cross-linked films were water-insoluble but permeable to water vapor. The water vapor transmission rate of chitosan films linearly decreased with an increasing concentration of the cross-linking agent; an optimum cross-linking agent concentration was found with alginate. The permeation and separation of CO_2 through a water-swollen chitosan membrane was studied. Carbon dioxide preferentially permeated through the swollen chitosan membrane with a permeability of 2.5×10^{-8} cm^3 (STP) cm and a CO_2/N_2 separation factor of 70 at room temperature. This separation performance for CO_2 resulted from the basic properties of the chitosan amino groups. The membrane preparation conditions, such as the acetic acid concentration of the casting solution, affected the membrane permeation rate (Ito et al. 1997).

34.2.2 Dialysis Membranes

Diffusion dialysis, as shown in Figure 34.2, can transport by simple diffusion according to the concentration gradient of solutes, those with a low molecular weight from a higher concentration solution side to the lower one, but not solutes with high molecular weight.

The effects of the kinds of acids used to dissolve chitosan, the chitosan concentration, the drying time of the dope solution, and the kinds of gelating agents acting on the membrane structure and their performance were studied in detail. On increasing the chitosan concentration, the solute permeability decreased while the selectivity of theophylline to vitamin B_{12} increased. The membrane changed from the wholly porous structure to the asymmetric structure by an increase of the chitosan concentration. Furthermore, the use of ethanol as the gelating agent brought about a wholly porous structure with a high permeability and a low selectivity. The asymmetric structure and the wholly dense structure were obtained in the cases of the gelating agents, such as the aqueous NaOH solution and dimethyl sulfoxide, respectively (Matsuyama et al. 1999a). The permeabilities of three kinds of solutes of similar sizes, such as anionic benzenesulfonic acid, neutral styrene glycol, and cationic theophylline in the chitosan membrane were investigated. Benzenesulfonic acid showed the highest permeability, whereas theophylline showed the lowest, although these solutes have almost the same size. This could be explained by the electrostatic attraction or repulsion between the solute and the membrane instead of the size-exclusion effect. The permeabilities of benzenesulfonic acid

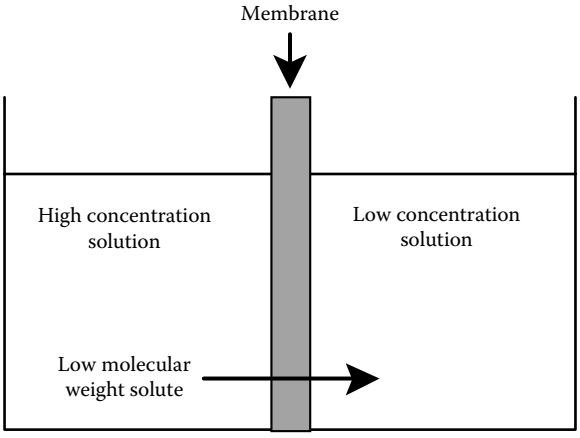


FIGURE 34.2 Principle of diffusion dialysis.

and theophylline increased and decreased, respectively, with the decrease of pH from 7.4 to 4.0 because of the increase of the charge density of the membrane. Thus, the selectivity of benzenesulfonic acid to theophylline increased and reached about 30 at pH 4.0 (Matsuyama et al. 1999b). In the chitosan and chitosan-poly (vinyl pyrrolodone) (PVP) membranes, the basic metabolites such as urea, creatinine, and glucose showed higher permeabilities than do the acidic metabolites, such as urea acid and phosphate (Qurashi et al. 1992). When an additional amount of PVP in the blend membranes was increased, the permeabilities increased. The permeability and the diffusion of vitamin B_{12} in the chitosan, chitosan cross-linked with glyoxal and gluteraldehyde, and chitosan/PVA membranes were studied using the "lag time" technique (Nakatsuka and Andrady 1992). The diffusion coefficient for both the cross-linked and blended chitosan membranes was solely dependent on the equilibrium swelling ratio of the membrane in water. The transport mechanism for vitamin B_{12} in these chitosan membranes was consistent with the "pore type." The equilibrium sorption of various sodium salts for chitosan, *N*-benzoylchitosan, and *N*-octanoylchitosan membranes was measured and explained by a dual mechanism, consisting of partition and Langmuir sorption (Seo et al. 1995). The mobility of anions through these membranes decreased with the Stokes radius and in the order chitosan > *N*-benzoylchitosan > *N*-octanoylchitosan membranes. The membranes immobilized prostaglandin E_1 on the heparin-modified chitosan/PVA blend membrane, which improved strength properties, permeability functions for low molecular weight solutes, and blood compatibility (Chandy and Sharman 1992). Organic-inorganic composite membranes from Chitosan (Chito) and tetraethoxysilane (TEOS) were prepared (Park et al. 2001). Drug permeation experiments were performed in a phosphate buffer solution of pH of 2.5 and 7.5, respectively. Lidocaine·HCl, sodium salicylate and 4-acetamidophenol were selected as model drugs to examine the effect of the ionic property of the drugs on permeation behavior. The effects of the membrane composition and the external pH on the swelling and the drug permeation behavior of Chito/TEOS membrane could be summarized as follows: chitosan incorporated into the Chito/TEOS membrane swelled at pH 2.5 while it shrunk at pH 7.5. This behavior was completely reversible and the membrane responded rapidly to the change in the environmental pH condition. According to the swelling behavior, an increase in pH from 2.5 to 7.5 yielded an increase in the permeation rate of the drug because of the shrinking of the incorporated chitosan in the Chito/TEOS membrane, while a decrease in pH resulted in a low permeation rate. The optimal Chito/TEOS ratio for maximum pH-sensitivity existed and the drug permeation was influenced not only by the external pH but also by the ionic interactions between the drug and the membrane.

34.2.3 Reverse Osmosis Membranes

When a solution containing solutes and only one solvent is set up across a semipermeable membrane, an osmotic pressure occurs. When a higher pressure than this osmotic pressure is applied on the side of the solution, only the solvent in the solution can be permeated from the solution side to the solvent through the semipermeable membrane and a solute in the solution can be rejected by the semipermeable membrane, as shown in Figure 34.3. This membrane-separation technique is called "reverse osmosis" and is applied for the desalination of seawater and brackish water.

The acetylated chitosan membrane (degree of acetylation: 72%) showed a high rejection and a high permeation rate in reverse osmosis for an aqueous solution of 0.2% $CaCl_2$, and had a high chemical reagent, an organic solvent, and heat stability (Adachi 1974).

Since chitosan molecules have amino and hydroxyl groups, and as the chitosan membrane was cross-linked with polyfunctional compounds such as dicarboxylic acid, diisocyanate, diglycigyl, and cyanul chloride, these cross-linked chitosan membranes were very stable for alkaline and acid solutions and were reverse osmosis membranes with a high water permeability and a high salt rejection. The resistance of novel surface cross-linked chitosan/poly(acrylonitrile) composite nanofiltration membranes to pH and organic solvents was studied with respect to the effects of cross-linking parameters, namely, glutaraldehyde concentration and cross-linking time. Pure water

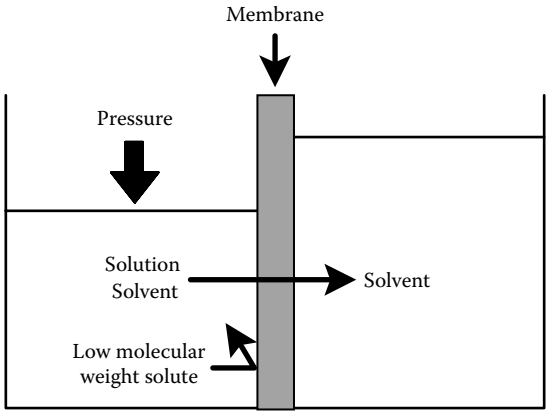


FIGURE 34.3 Principle of reverse osmosis.

flux and polyethylene glycol transmission data indicated that at pH 2.5 and 11, the membrane stability increased with increasing glutaraldehyde concentration and was much better at pH 11 than at pH 2.5. All surface cross-linked membranes showed a reduced swelling between pH 4 and 10 (Musale and Kumar 2000).

34.2.4 Ultrafiltration Membranes

The principle of ultrafiltration is shown in Figure 34.4, in which the low molecular weight solutes, such as inorganic salts and organic low molecular weight compounds can be permeated with the solvent through an ultrafiltration membrane, but the high molecular weight solutes such as protein and polysaccharide cannot. In ultrafiltration, since the separable materials by ultrafiltration are polymer solutes, the osmotic pressure is much lower than that in reverse osmosis. Consequently, in general, the operating pressure is about 5–50 pa.

Chitin ultrafiltration membranes were prepared by the wet method using DMA/NMP/LiCl as a casting solvent and water as a gelation medium (Uragami et al. 1981). These membranes were asymmetric porous structures and the ultrafiltration characteristics for an aqueous solution of PEG 6000 were significantly influenced by the temperature during membrane preparation. These results are attributed to the absorption of water from the atmosphere into the casting solutions during the casting process. Because DMA and NMP are solvents with very low volatility and

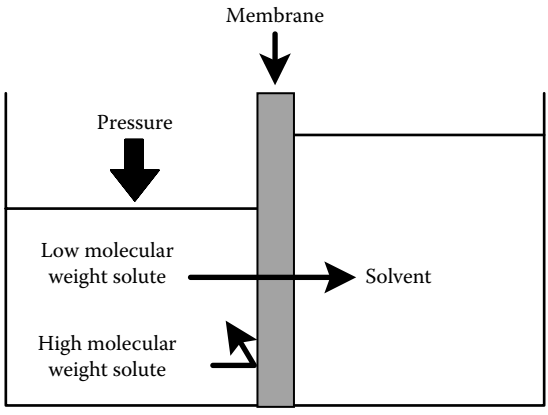


FIGURE 34.4 Principle of ultrafiltration.

very high hygroscopicity and since LiCl is deliquescent, the resulting membrane structures are affected by the temperature during membrane preparation. To obtain ultrafiltration membranes for blood treatment, as dried quaternized chitosan membranes were immersed into ethyleneglycol diglycisyl ether (EGDGE) containing a small amount of NaOH, the hydroxyl groups in the quaternized chitosan molecules were cross-linked with EGDGE and water-insoluble quaternized chitosan membranes were obtained. When these water-insoluble membranes were again immersed into an aqueous solution of sodium heparin, polyion complexes between quaternized chitosan and sodium heparin were formed and consequently, heparinized chitosan membranes could be prepared (Uragami et al. 1988a). These heparinized chitosan membranes could perfectly permeate low molecular weight solutes such as urea, creatinine, and vitamin B_{12} and completely block protein such as albumin, and also showed an excellent antithrombogenicity in an *in vivo* test (Uragami et al. 1988). Five proteins, namely ovalbumin, human serum albumin, soybean trypsin inhibitor, lysozyme, and cytochrome *C* were selected as model proteins to investigate their adsorption on the chitosan membranes with controlled poresizes. These proteins could be efficiently recovered (91%–98%) from the membranes using 1 N NaCl in a 0.02 N sodium phosphate solution (pH 6) as eluant. Protein separations were performed from binary mixtures (ovalbumin-lysozyme, human serum albumin-cytochrome *C*, and soybean trypsin inhibitor-cytochrome *C*), and high purity products (similar to 99%) were obtained in a single pass (Zeng and Ruckenstein 1998).

34.2.5 Pervaporation Membranes

In pervaporation, as shown in Figure 34.5, when feed mixtures are added on one side of the membrane and the other side is evacuated, a certain component in the feed mixture can be preferentially permeated through the membranes (Binning et al. 1961; Choo 1962). In the pervaporation, the difference in the solubility of permeants into the membrane, the diffusivity of permeants in the membrane, and the relative volatility of permeants from the membrane can influence the characteristics of permeation and separation. This pervaporation technique is advantageous for the separations of azeotropic mixtures, close-boiling point mixtures, and structural isomers.

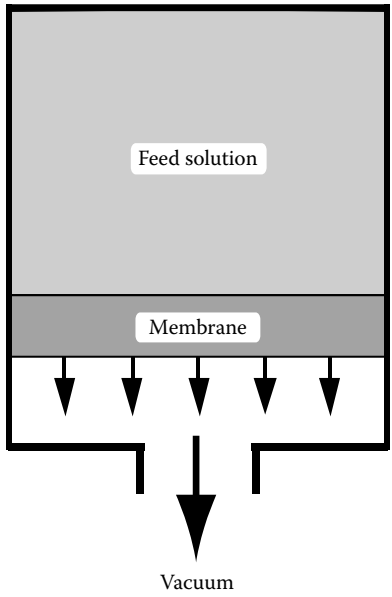


FIGURE 34.5 Principle of pervaporation.

34.2.5.1 Water/Organic Selective Membranes

Chitosan is a hydrophilic polymer such as cellulose, alginic acid, and pullulan. The characteristics of permeation and separation for an aqueous alcohol solution of the chitosan membrane in pervaporation is summarized in Table 34.1, in which separation factors, $\alpha_{H_2O/ROH}$ was calculated by

$$\alpha_{H_2O/ROH} = \frac{(Y_{H_2O}/Y_{ROH})}{(X_{H_2O}/X_{ROH})} \tag{34.1}$$

where X_{H_2O}, X_{ROH}, Y_{H_2O}, and Y_{ROH} denote the weight fractions of water and alcohol in the feed solution and in the permeate, respectively.

The permeation rate for all aqueous solutions decreased with an increasing alcohol concentration in the feed solutions. In aqueous solutions of methanol, which has a relatively small molecular size and high volatility, the values of the separation factor were small. Also, for aqueous 1-propanol solutions in pervaporation, the separation factors were higher and increased significantly with the content of 1-propanol in the feed. From the results, it is suggested that the concentration of aqueous alcoholic solutions through the chitosan membrane is more effective for aqueous solutions with a higher alcohol content than for those with a lower alcohol content (Uragami et al. 1988). A composite chitosan membrane coated onto a porous polysulfone (PS) support membrane had a higher membrane performance of the pervaporation dehydration of 2-propanol/water more than a homogeneous membrane for the feed with a high 2-propanol content (Wang et al. 1996). The permeation rate increased and the separation factor decreased with an increase of the permeation temperature. The homogeneous chitosan membrane cross-linked with hexamethylene diisocyanate improved the water/ethanol selectivity but reduced the permeation rate. A thin, dense chitosan membrane coated onto a hydrolyzed poly (acrylonitrile) (PAN) support membrane had a good permeation rate of 0.26 kg/m^{2}h and a high separation factor of more than 8000 for an aqueous solution of 90 wt% ethanol at 60°C, and 0.8 kg/m^{2}h and 1 kg/m^{2}h in the permeation rate for an aqueous solution of 80 wt% of 1-propanol and 2-propanol, respectively and the separation factor of about 10^5 (Ghazuhi et al. 1997). To improve a higher water/ethanol selectivity of the chitosan membrane, the chitosan membranes were chemically modified (Uragami and Takigawa 1990). Pervaporation properties for the aqueous ethanol solution of the chitosan membranes with different deacetylation were studied (Miya et al. 1985). The permeation rate is increased with increasing water content of the chitosan

TABLE 34.1
Permeation and Separation Characteristics for Aqueous Alcohol Solutions through the Chitosan Membrane during Pervaporation

Methanol			Ethanol			1-Propanol	
Feed (wt%)	Permeation Rate (10^3 kg/m^2 h)	($\alpha_{H_2O/MeOH}$)	Feed (wt%)	Permeation Rate (10^3 kg/m^2 h)	($\alpha_{H_2O/MeOH}$)	Feed (wt%)	Permeation Rate (10^3 kg/m^2 h)
0	18.6	—	0	186.0	—	0	337.4
10	15.0	0.7	10	150.0	0.7	10	111.0
30	16.0	1	30	136.0	2	30	141.7
50	9.4	1	50	67.1	13	50	106.2
70	6.8	2	70	34.6	50	71.8[a]	50.5[a]
90	4.3	2	90	12.3	31	90	8.2
		—	96.5[a]	6.5[a]	17[a]		
100	2.4		100	2.9	—	100	1.3

[a] Azeotropic composition.

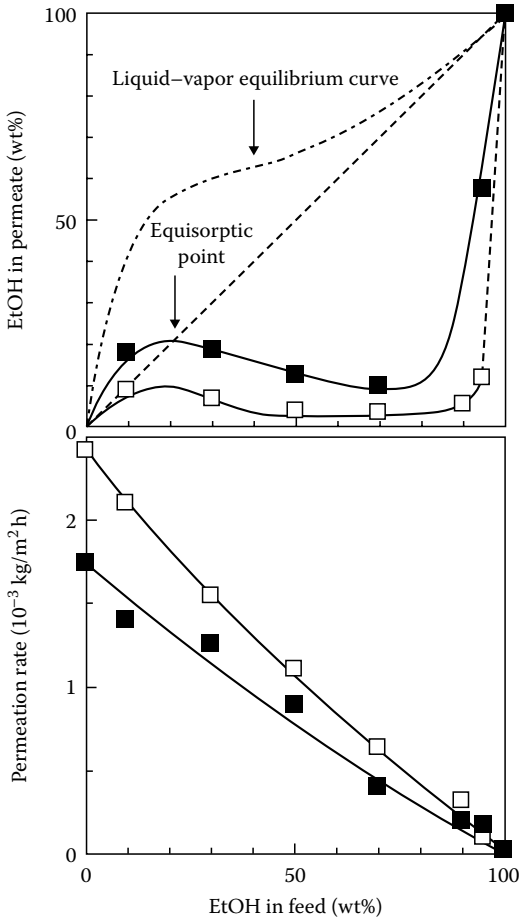


FIGURE 34.6 Characteristics of permeation and separation for aqueous ethanol solutions through the chitosan (■) and GAC (□) membranes in pervaporation. (From Uragami, T. and Takigawa, K., *Polymer*, 31, 668, 1990.)

membrane but the separation factor for the water/ethanol selectivity had a maximum at a water content of about 25%. The permeation and separation characteristics of ethanol/water mixtures through the chitosan membrane and the glutaraldehyde cross-linked chitosan (GAC) membrane by pervaporation is shown in Figure 34.6 (Uragami and Takigawa 1990).

The chitosan and GAC membranes predominantly permeated water. Also, azeotropic compositions, namely 96.5 wt% ethanol in the feed solution, were not observed in either membranes. In the chitosan membrane, an equisorpic composition was found. It implies that the separation cannot be entirely obtained through the membrane. An appearance of the equisorptic point is attributed to a remarkable swelling of the chitosan membrane due to the feed mixture. The chitosan membrane in pervaporation selectively permeates ethanol in the feed solution with a low ethanol concentration. A selective permeation of ethanol in the feed with a low ethanol concentration is due to the fact that the relative volatility of ethanol gives a greater effect on separation in the evaporation process than in separation during the diffusion process. However, the equisorptic point disappeared in the GAC membrane. This fact suggests that the swelling of the membrane, due to the aqueous solution with a low ethanol concentration, is significantly prevented by cross-linking the chitosan membrane with glutaraldehyde. The permeation rates through GAC membranes were greater than those for the chitosan membranes. This tendency increased with the decrease of the ethanol concentration in the feed mixture. The separation characteristics of the GAC membrane were higher

than those of the chitosan membrane. The fact that the GAC membrane had higher permeation rates and separation characteristics than those for the chitosan membrane may be interpreted as follows. When the polymer membrane is cross-linked, in general, the resulting membrane becomes denser, and the separation characteristics of this membrane increase but the permeation rate decreases. However, the GAC membrane increases both the permeation rate and the separation characteristics. In this study, the chitosan membrane was cross-linked in an aqueous solution with glutaraldehyde. Therefore, the chitosan membrane is swollen in an aqueous solution and is then cross-linked with glutaraldehyde. Consequently, the dense GAC membrane with a high hydrophilicity is formed. A higher permeation rate and separation characteristics for the GAC membrane depend on both the dense structure and the high hydrophilicity of the GAC membrane. Hydrophilic organic-inorganic hybrid membranes were prepared from hydrophilic quaternized chitosan (q-Chito) and tetraethoxysilane (TEOS) by the sol-gel process, in order to lower the swelling of q-Chito membranes. When an aqueous solution of 96.5 wt% ethanol was permeated through the q-Chito/TEOS hybrid membranes during pervaporation, the q-Chito/TEOS hybrid membranes showed a high water/ethanol selectivity. However, the water/ethanol selectivity of the membranes decreased slightly with increasing TEOS content of over 45 mol%. Furthermore, the water/ethanol selectivity of the membranes is discussed from the viewpoint of chemical and physical membrane structures (Uragami et al. 2004). Cross-linked organic-inorganic hybrid chitosan membranes were prepared by blending chitosan and γ-(glycidyloxypropyl)trimethoxysilane (GPTMS) in an aqueous solution of acetic acid (Liu et al. 2005). The hydrophilicity of the modified membranes was not significantly decreased so as to result in good water selectivity and high permeation rate in pervaporation on a 70 wt% isopropanol/water mixture A permeation rate of 1730 $g/m^2 \cdot h$ and a separation factor, $\alpha_{H_2O/IPA}$ of 694 were found with the chitosan membrane containing 5 wt% GPTMS. Both, the cross-linked and the organic-inorganic hybrid structures contribute to stabilizing the membrane to maintain the performance of the membranes in a 140 day long-term operation.

34.2.5.2 Alcohol/Water Selective Membranes

The pervaporation behavior of aqueous ethanol mixtures through the poly(ethylene oxide) (PEO)/chitosan (Chito) blend membrane was investigated. The results showed that both the Chito and the PEO/Chito membrane preferentially permeate ethanol at a lower alcohol concentration in feed, and the selectivity of the Chito membrane toward alcohol can be greatly improved by introducing the hydrophilic polymer PEO into the Chito. The PEO/Chito blend membrane gave the separation factor, α_{EtOH/H_2O} was 4.4, and the permeation rate was 0.9 $kg/m^2 \cdot h$ for 8 wt% of ethanol in the feed (Wang et al. 1999).

34.2.5.3 Organic/Organic Separation Membranes

Benzoylchitosans (BzCses) with a different degree of benzoylation were synthesized as membrane materials with a good durability for the separation of benzene/cyclohexane (Bz/Chx) mixtures. The BzCs membranes showed a high benzene permselectivity for a Bz/Chx mixture of 50 wt% benzene in pervaporation and the difference of the benzene permselectivity for the BzCs membranes with a different degree of benzoylation corresponded to a difference in the physical structure of the membranes based on the characteristics of these membranes. When a Bz/Chx mixture of 50 wt% benzene was permeated through the BzCs membranes, the permeation rate increased and the Bz/Chx selectivity slightly decreased with an increasing degree of benzoylation, as shown in Figure 34.7 (Inui et al. 1998, Uragami et al. 1998). For the pervaporation separation of ethanol/toluene and methanol/toluene mixtures, chitin membranes composited onto a porous polyetherimide support were prepared. The incorporation of additional acetyl groups into the chitosan structure decreased the total permeation rate and increased the separation factor from 401 $g/m^2 \cdot h$; 34 (pure chitosan) to 282 $g/m^2 \cdot h$; 116 (4 mol acetylated chitosan) for 10% of EtOH feed mixture and from 681 $g/m^2 \cdot h$; 159 (pure chitosan) to 484 $g/m^2 \cdot h$; 607 for 10% of the MeOH feed mixture (Huang et al. 2000). The pervaporation separation of methanol/methyl tertiarybutyl ether (MeOH/MTBE) mixtures was

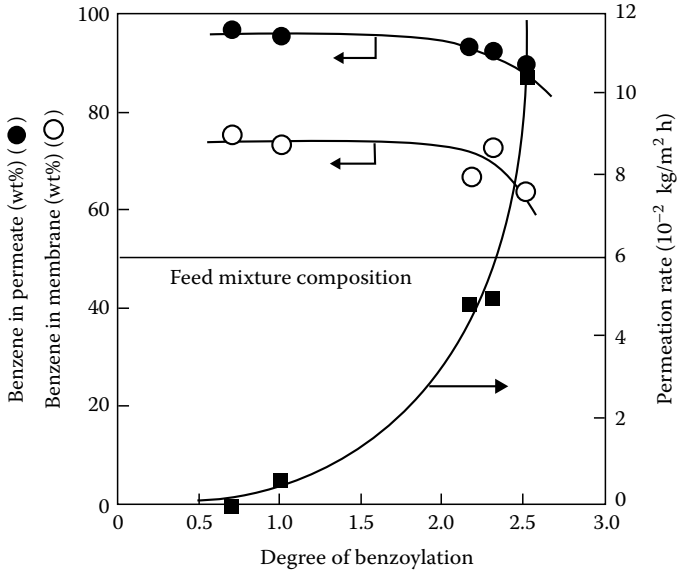


FIGURE 34.7 Effects of the degree of benzoylation on the benzene concentration in the permeate (●) and permeation rate (■) through the benzoylcellulose membranes for a benzene/cycloxexane of 50 wt% benzene by pervaporation and the composition of benzene/cycloxexane sorbed into their membranes (○) for a benzene/cycloxexane mixture. The dotted line is the feed mixture composition (benzene/cycloxexane = 50/50, w/w). (From Inui, K. et al., *J. Membr. Sci*., 138, 67, 1998.)

accomplished through chitosan composite membranes modified with sulfuric acid and four surfactants. The chitosan composite membrane modified with sulfuric acid showed a pervaporation performance of over 70 wt% methanol in the permeate and the permeation rate was 100 g/m^2·h. For the membrane complexed with surfactants, the permeate showed a 98.3 wt% methanol concentration and a 470 g/m^2·h permeation rate. With an increasing operating temperature, the permeate flux remarkably increased to 1170 g/m^2·h and the permeate showed a 97.8 wt% methanol concentration (Nam and Lee 1999). Chitosan with poly(*N*-vinyl-2-pyrrolidone) (PVP) blend membranes were evaluated for the separation of methanol from MTBE. MeOH preferentially permeated through all the tested membranes, and the partial flux of methanol significantly increased with increasing PVP content. The temperature dependence of pervaporation performance indicated that a significant conformational change occurred with increasing temperature (Cao et al. 1999). For the separation of MeOH/MTBE mixtures, methanol selective chitosan composite membranes were prepared. Pervaporation characteristics of surfactant-modified chitosan membranes were substantially improved due to the decreased membrane thickness and the possible enhanced affinity to methanol (Huang et al. 2001). Polyion complex composite membranes were prepared from sodium alginate and chitosan. These membranes showed an excellent pervaporation performance in the separation of MeOH/MTBE mixtures. In particular, a membrane prepared from a 2.0 wt% sodium alginate solution and a 2.0 wt% chitosan solution appeared to permeate only MeOH from the feed, with a permeation rate of over 240 g/m^2·h. As the operating temperature increased from 40°C to 55°C, the permeation rate of MeOH increased, but that of MTBE decreased (Kim et al. 2000).

34.2.6 Evapomeation Membranes

An "evapomeation" method as a new membrane separation technique, which makes use of the advantage of pervaporation and simultaneously removes a fault of pervaporation, was developed (Uragami et al. 1988b; Uragami et al. 1989b). In this evapomeation technique, the feed solutions are fed without direct contact with the polymer membrane, and only vapor is supplied to the polymer

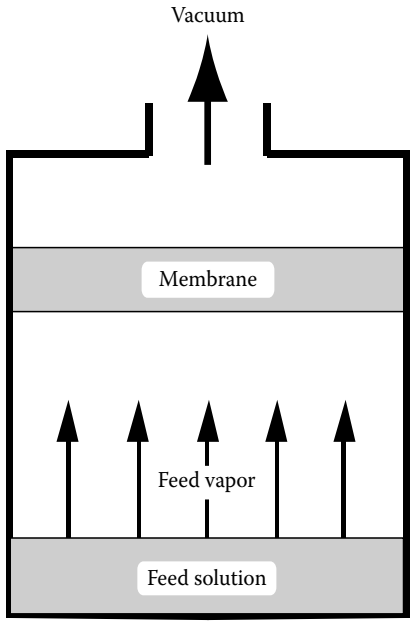


FIGURE 34.8 Principle of evapomeation. (From Uragami, T. et al., *Chem. Rapid Commun*., 9, 361, 1988.)

membrane and the swelling or shrinking of polymer membranes due to the feed solutions are prevented, as shown in Figure 34.8.

The permeation and separation characteristics for various aqueous ethanol solutions through the chitosan membrane by evapomeation and pervaporation are compared in Table 34.2 (Uragami et al. 1988b).

Both the permeation rate in evapomeation and pervaporation decreased with an increase of the ethanol concentration in the feed. These results suggest that the chitosan membrane becomes dense as the ethanol content in the feed is increased. The permeation rates in evapomeation were smaller by one order of magnitude as compared with those in pervaporation. This fact supports the

TABLE 34.2
Permeation and Separation Characteristics for Aqueous Ethanol Solutions through the Chitosan Membrane during Pervaporation and Evapomeation

	Pervaporation		Evapomeation	
C_2H_5OH in Feed (wt%)	Permeation Rate (10^2 kg/m^2 h)	Separation Factor ($\alpha_{H_2O/EtOH}$)	Permeation Rate (10^2 kg/m^2 h)	Separation Factor ($\alpha_{H_2O/EtOH}$)
0	186.0		176.0	
10 (43.9)	150.0	0.7	148.0	5 (33)
30 (60.4)	136.0	2	126.0	7 (25)
50 (67.7)	67.1	13	95.6	26 (56)
70 (77.3)	34.6	50	39.2	37 (53)
90 (90.8)	12.3	31	18.0	114 (124)
96.5[a]	6.5	17	7.3	202
100	2.9		6.2	

Note: Value in parentheses are for vapor compositions.

[a] Azeotrope.

assumption that the chitosan membrane in evapomeation can almost keep the dense structure existing prior to the evapomeation experiment and that consequently, the diffusivity of the permeating species during the diffusion process is lowered.

As can be seen from this table, water was predominantly permeated through the chitosan membrane both in pervaporation and evapomeation. An azeotropic composition, viz. 95.6 wt% ethanol in the feed solution, was not observed in both methods, and the water/ethanol selectivities in evapomeation were greater than those in pervaporation. These results depend on the fact that the swelling of the chitosan membrane in evapomeation is remarkably prevented as compared with that in pervaporation. The effect of the feed-vapor composition of the aqueous ethanol solutions on the permeation rate, the ethanol concentration in the permeate through the chitosan and GAC membranes in evapomeation, and the degree of swelling of the membrane is shown in Figure 34.9 (Uragami et al. 1994).

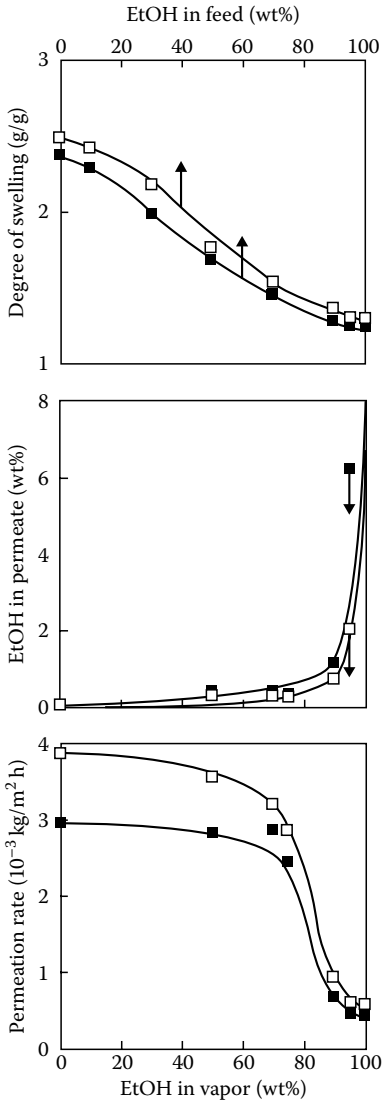
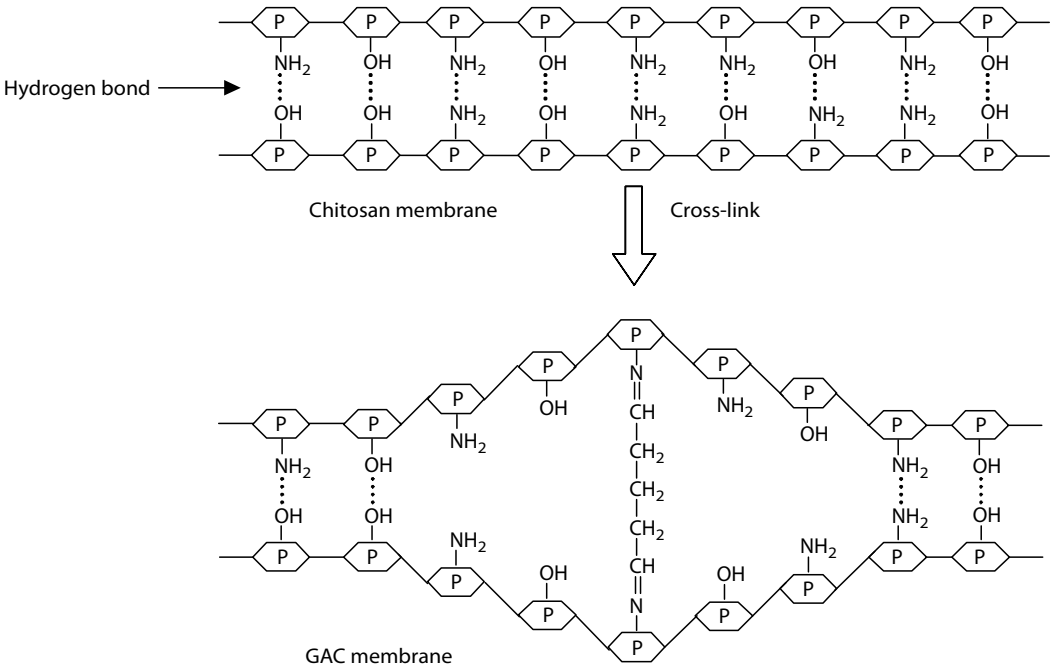


FIGURE 34.9 Effects of ethanol concentration in the feed vapor on the permeation and the separation characteristics and the degree of swelling of the chitosan (■) and GAC (□) membranes by evapomeation. (From Uragami, T. et al., *J. Membr. Sci.*, 88, 243, 1994.)

The GAC membrane had a content of 0.5% glutaraldehyde (3.2% glutaraldehyde in the casting solution). Both the chitosan and GAC membranes had a low ethanol concentration in the permeate and showed a high water/ethanol selectivity. Also, there are significant differences between the compositions in the permeate of the chitosan and the GAC membrane was higher than that of the chitosan membrane in spite of the fact that the permeation rate of the GAC membrane was greater than that of the chitosan membrane. Furthermore, the degree of swelling of the GAC membrane was higher than that of the chitosan membrane and this tendency increased with a decreasing ethanol concentration in the feed mixture. When polymer membranes are cross-linked, in general, the permselectivity is improved but the permeation rate is decreased because the degree of swelling of the membrane is lowered. In this case, however, the permeation rate, the water/ethanol selectivity, and the degree of swelling of the GAC membrane are higher than those of the chitosan membrane. In order to clarify the results in Figure 34.9, the density and the crystallinity of chitosan and the GAC membranes were determined by the flotation method and wide-angle x-ray diffraction, respectively. The density and the correlation crystallinity index decreased with an increasing glutaraldehyde content in the casting solution. These results imply that the increase in the cross-linking of the chitosan membrane decreases the density and the crystallinity of the membrane. From these results, a model structure, as shown in Scheme 34.1, is assumed for the chitosan and GAC membranes.

The chitosan membrane has many intermolecular hydrogen bonds between hydroxyl groups and amino groups. A few of these hydrogen bonds in the GAC membrane are broken by cross-linking with glutaraldehyde and free hydrophilic groups such as hydroxyl, and amino groups are formed. These hydrophilic groups have a strong affinity to water molecules, i.e., the solubility of water molecules into the GAC membrane is increased. On the other hand, since the size of the water molecule sorbed into the GAC membrane is smaller than that of the ethanol molecule, the water molecule can be easier diffused in the GAC membrane than the ethanol molecule. Consequently, the GAC membranes are moderately swollen by water molecules and simultaneously increase the water/ethanol selectivity. The increase of the water/ethanol selectivity in the GAC membrane is due to both the increase in the solubility of water molecules into the GAC membrane and the increase in the diffusivity of water



SCHEME 34.1 Model structures of the chitosan and GAC membranes. ⟨G⟩: glucosamine unit, —: hydrogen bond. (From Uragami, T. et al., *J. Membr. Sci*., 88, 243, 1994.)

molecules in the GAC membrane. From the above discussion, both the increase in the permeation rate and the separation factor with increasing glutaraldehyde content cross-linked in the chitosan membrane can be understood. If a deformation of the hydrogen bonds in the chitosan membrane, as shown in Scheme 34.1, gives a high permeation rate and high water/ethanol selectivity, it can be assumed that cross-links are not necessarily needed. So a chemical modification of the chitosan membrane was tried using *N*-alkyl aldehyde as a monofunctional aldehyde and these *N*-alkyl chitosan membranes were applied to the permeation and the separation for aqueous ethanol solutions in evapomeation (Uragami et al. 1997). If a balance between an increase of the hydrophilicity of the *N*-alkyl chitosan membranes based on the deformation of the hydrogen bonds in the chitosan membrane and an increase of the hydrophobicity of the membranes due to the *N*-alkylation of the amino groups in the chitosan membrane are suitable, both the permeation rate and the water/ethanol selectivity are improved by the alkylation of the chitosan membrane.

Glutaraldehyde cross-linked chitosan acetate (GA-ChitoA) and cross-linked carboxymethyl chitosan acetate (GA-CM-ChitoA) membranes showed a high water permselectivity for aqueous alcohol solutions in evapomeation (Uragami et al. 1993b). The high water permselectivity for the GA-CM-ChitoA membrane was attributed both a high solubility of the water molecules into the membrane due to a high hydrophilicity of GA-CM-ChitoA and an inhibition of diffusivity of the ethanol molecules with larger molecular size based on the introduction of bulky carboxymethyl groups. The water/ethanol selectivity through the GA-CM-ChitoA membrane in evapomeation was in the order of aqueous solutions of methanol < ethanol < 1-propanol.

The permeation and the separation characteristics during evapomeation of an ethanol/water azeotrope (96.5 wt% ethanol) through quaternized chitosan (q-Chito) membranes and cross-linked q-Chito membranes, which were cross-linked with diethylene glycol diglycidyl ether (DEDGE), were studied (Uragami et al. 2002a). Both the q-Chito and the cross-linked q-Chito membranes showed a high water/ethanol selectivity for an ethanol/water azeotrope. The permeation rates for both membranes decreased and the water permselectives increased, with an increasing degree for the quaternization of the chitosan and the cross-linker concentration. The mechanism of separation for the ethanol/water azeotrope through the q-Chito and the cross-linked q-Chito membranes was analyzed by the solution-diffusion model. An increasing permeation temperature increased the permeation rate and decreased the water/ethanol selectivity for both membranes. However, the permeation rates of a cross-linked q-Chito membrane at 60°C–80°C were almost the same as those of the q-Chito membrane, and the separation factors for the water/ethanol selectivity ($\alpha_{sepH2O/EtOH}$) = 4100–4200) in the former were greater by two orders of magnitude as compared to the latter ($\alpha_{sepH2O/EtOH}$) = 47–58). Dehydration of an ethanol/water azeotrope during evapomeation using polyion complex cross-linked chitosan composite (q-Chito-PEO acid polyion complex/PES composite) membranes, constracted from the quaternized chitosan (q-Chito) and the polyethylene oxydiglycilic acid (PEO acid) on a porous polyethersulfone (PES) support, was investigated (Uragami et al. 1999, 2003). Both the q-Chito/PES composite and the q-Chito-PEO acid polyion complex/PES composite membranes showed a high water/ethanol selectivity for an ethanol/water azeotrope. Both the permeation rate and the water/ethanol selectivity of the q-Chito/PES composite membranes were enhanced by increasing the degree of quaternization of the chitosan molecule, because the affinity of the q-Chito/PES composite membranes for water was increased by introducing a quaternized ammonium group into the chitosan molecule. Q-Chito-PEO acid polyion complex/PES composite membranes prepared from an equimolar ratio of carboxylate groups in the PEO acid versus quaternized ammonium groups in the q-Chito showed the maximum separation factor for the water/ethanol selectivity without lowering the permeation rate, as shown in Figure 34.10.

With an increasing molecular weight of PEO acid, the separation factor for the water/ethanol selectivity increased, but the permeation rate almost did not change. The permeation rate, separation factor for the water/ethanol selectivity, and the evapomeation index of the q-Chito-PEO acid 400 polyion complex/PES composite membrane with an equimolar ratio of carboxylate groups in PEO

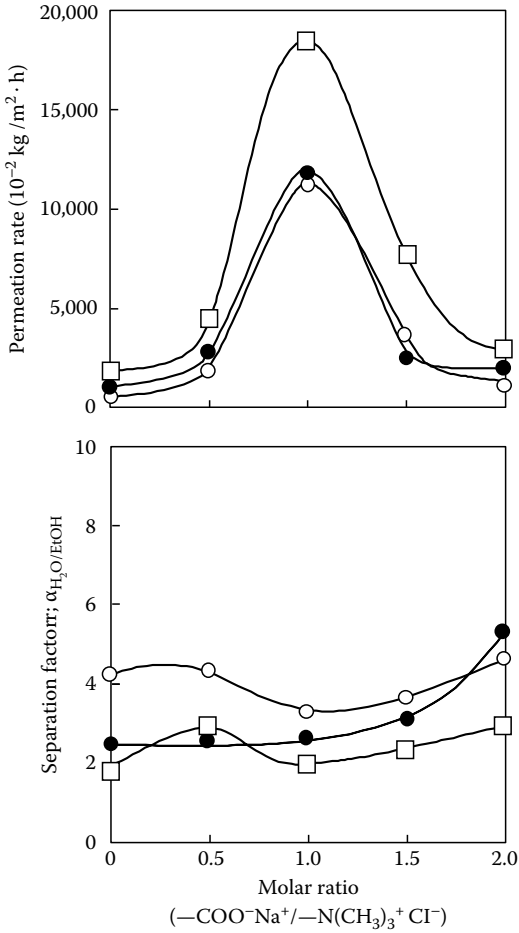


FIGURE 34.10 Effects of the molar ratio of the carboxylate versus the ammonium groups on the permeation rate and the separation factor for water for an aqueous solution of 96.5 wt% ethanol (○), 1-propanol (●), and 2-propanol (□) through the q-Chito–PEO acid 4000 polyion complex/PES composite membrane during evapomeation at 60°C. (From Uragami, T. et al., *Biomacromolecules*, 4, 137, 2003.)

acid 400 and ammonium groups in q-Chito were 3.5×10^{-1} kg/m^2·h 6300, and 2205, respectively. The separation factor for the water/ethanol selectivity for aqueous solutions of 1- and 2-propanol was also maximized at an equimolar ratio of carboxylate groups and ammonium groups and was greater than for an ethanol/water azeotrope. Two kinds of cross-linked quaternized chitosan (q-chito) membranes were prepared for the dehydration of ethanol/water mixtures. One was prepared by coating a casting solution of q-chito containing glutaraldehyde (GA) with a HCl catalyst onto a porous polyethersulfone (PES) support (membrane A). The other was membrane A further cross-linked in an aqueous GA solution with a H_2SO_4 catalyst (membrane B). These membranes were then applied to the dehydration of ethanol/water mixtures for high temperature and high pressure evapomeation (HTPEV). The permeation rate increased with the increasing feed vapor pressure, but decreased with an increase in the feed vapor temperature under constant feed vapor pressure. This decrease in the permeation rate could be attributed to a lowering of the vapor density, which is the ratio of the feed vapor pressure over the total pressure (P_1/P_T). The permeation rate derived from the relationship equation, which was driven statistically by the experimental data with a change in the feed vapor temperature under constant feed vapor pressure, agreed closely with that predicted from the equation as a function of the ratio of P_1/P_T. The separation characteristics for water permselectivity

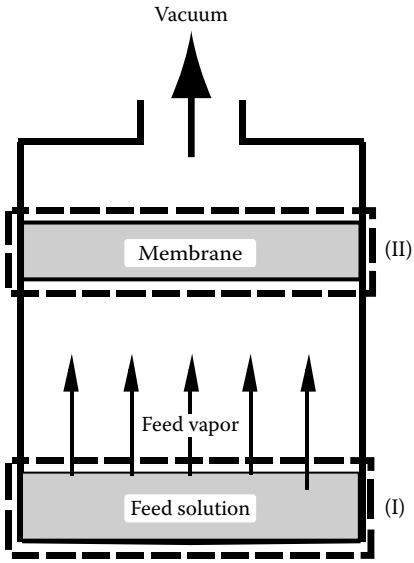


FIGURE 34.11 Principle of temperature difference controlled evapomeation (TDEV). (From Uragami, T. and Morikawa, T., *Makromol. Chem. Rapid Commun*., 10, 28, 1989.)

in the water/ethanol vapor increased with a decrease in the difference between P_T and P_1 (Uragami and Tokura 2006).

In this evapomeation, the temperatures of the feed solution (I) and the membrane surroundings (II) are controlled and consequently a difference between these temperatures can be established, as shown in Figure 34.11. Such an evapomeation method with a control of the temperature difference is named "temperature-difference controlled evapomeation, TDEV" and is a new membrane separation technique (Uragami and Morikawa 1989; Uragami and Shinomiya 1991).

The characteristics of permeation and the separation for an aqueous dimethyl sulfoxide (DMSO) solution through the chitosan membrane by TDEV are shown in Figure 34.12, in which the feed was an aqueous solution of 50 wt% dimethyl sulfoxide, the temperature of the feed solution was kept constant at 40°C and the temperature of the membrane surroundings was changed to a temperature less than the temperature of the feed solution (Uragami and Shinomiya 1992).

Both the total permeation rate and the separation factor increased with a decreasing temperature of the membrane surroundings. The increase of the total permeation rate may be due to the increase of the solubility of vapor in the membrane with a drop of the temperature of the membrane surroundings according to Henry's law. The increase of the separation factor, i.e., an improvement of the H_2O/DMSO selectivity, is explained by the illustration shown in Figure 34.13.

When the dimethylsulfoxide and the water molecules that vaporize from the feed mixture come close to the membrane surroundings, the dimethyl sulfoxide vapor aggregates much easier than the water vapor (because the freezing points of dimethyl sulfoxide and water are 18.4°C and 0°C, respectively), and tends to liquefy as the temperature of the surroundings of the membrane becomes lower. This aggregation of the dimethyl sulfoxide molecules is responsible for the increase of the H_2O/DMSO selectivity through the chitosan membrane. The increase in the separation factor with the TDEV method, in which the temperature of the membrane surrounding is lower than the temperature of the feed solution, is attributed to the influence of the degree of aggregation of the dimethyl sulfoxide molecule on the membrane surroundings, which is significantly governed by the temperature of the membrane surroundings. The high H_2O/DMSO selectivity of the chitosan membrane for aqueous dimethyl sulfoxide solutions in TDEV is significantly enhanced by both the high affinity for water of the chitosan membrane and the decrease of the solubility selectivity for dimethyl sulfoxide molecules into the chitosan membrane based on their aggregation on the membrane

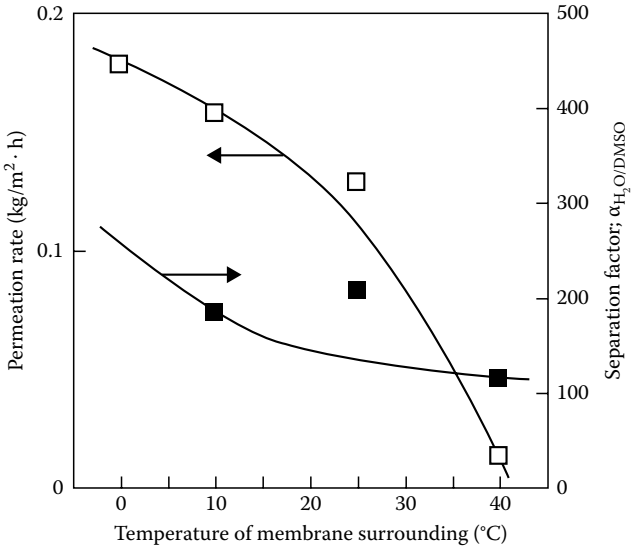


FIGURE 34.12 Effect of the temperature of the membrane surroundings on the characteristics of permeation and separation for an aqueous solution of 50 wt% dimethyl sulfoxide through the chitosan membrane in TDEV. Feed temperature: 40°C. (From Uragami, T. and Shinomiya, H., *J. Membr. Sci*., 74, 18, 1992.)

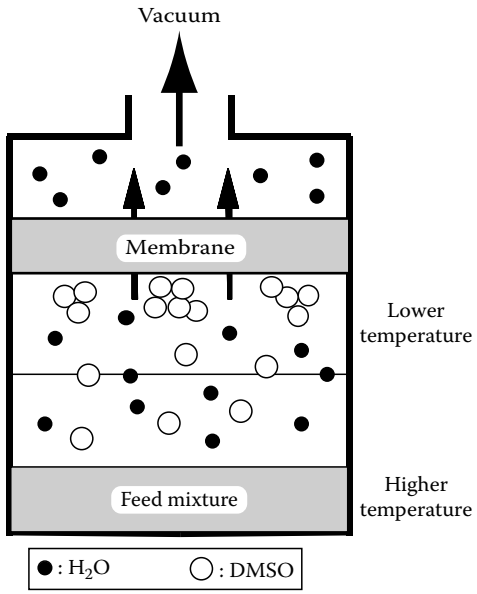


FIGURE 34.13 Tentative separation mechanism for aqueous dimethyl sulfoxide solutions through the chitosan membrane in TDEV. (From Uragami, T. and Shinomiya, H., *J. Membr. Sci*., 74, 18, 1992.)

surroundings. The permeation rate for water and dimethyl sulfoxide for an aqueous solution of 50 wt% dimethyl sulfoxide through the chitosan membrane in TDEV are shown in Figure 34.14, in which the temperature of the membrane surroundings was changed.

As can be seen from these results, the permeation rate for dimethyl sulfoxide were remarkably low compared with those for water, i.e., it was about one hundred thousandth. The difference

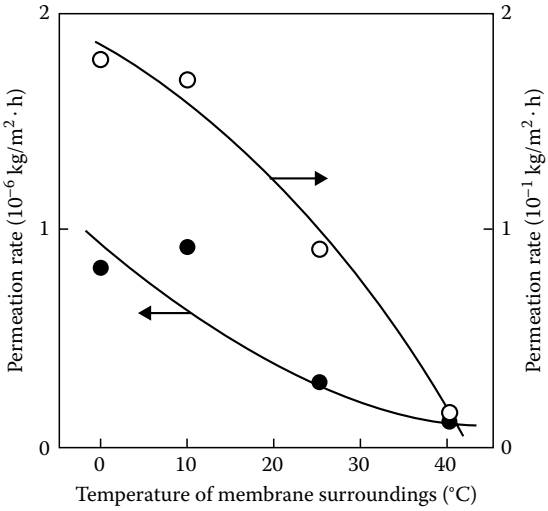


FIGURE 34.14 Permeation rates for water (○) and dimethyl sulfoxide (●) for an aqueous solution of 50 wt% dimethyl sulfoxide through the chitosan membrane in TDEV as a function of the temperature of the membrane surroundings. Feed temperature: 40°C. (From Uragami, T. and Shinomiya, H., *J. Membr. Sci*., 74, 18, 1992.)

between the permeation rate for water and dimethyl sulfoxide became larger with a lowering of the temperature of the membrane surroundings. These results support the discussion of the mechanisms for permeation and separation in TDEV described above.

34.2.7 Carrier Transport Membranes

Material transport through membranes, in general, is classified into three fundamental types, as shown in Figure 34.15 (Uragami 1992). Figure 34.15a is a model for the passive transport that transfers the material, S, from the left side (B side), with high concentration, to the right side (A side), with low concentration, according to its concentration gradient across the membrane.

In Figure 34.15b, the carrier, C, in the membrane positively incorporates the material, S, into the membrane by forming a complex, CS, between C and S. In this transport system, in addition to the passive transport in Figure 34.15a, transport with the formation of the complex is added. Consequently, since the transport of material is facilitated, it is called facilitated transport. In such transport, if the carrier can form a complex with a specific material, selectively facilitated transport is possible. In both Figure 34.15a and b, the material could be transported from the high-concentration

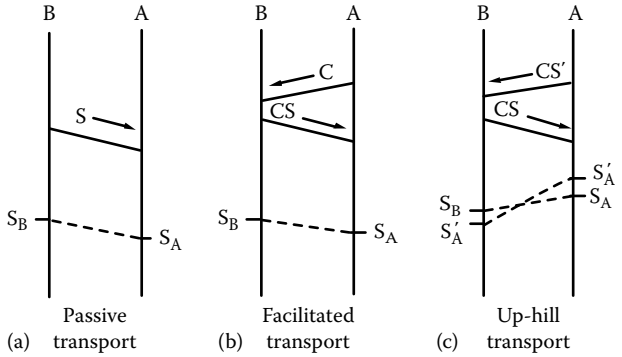


FIGURE 34.15 Fundamental types of the membrane transport.

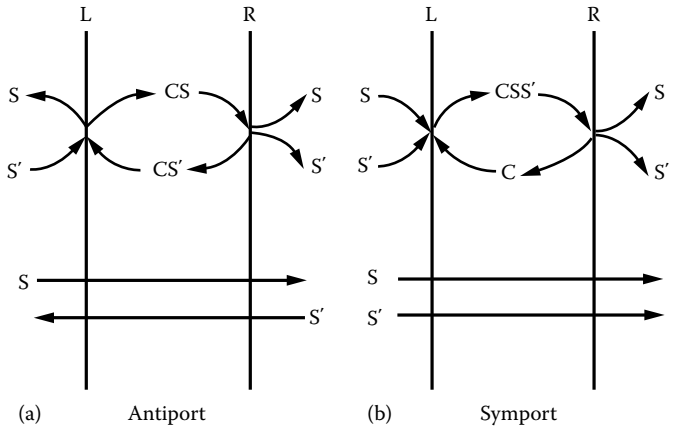


FIGURE 34.16 Types of uphill transport.

side (B side) to the low side (A side) but is never transported because the concentrations are equal on the two sides of the membrane.

A model of an uphill transport is shown in Figure 34.15c. In this case the material is actively transported from the low-concentration side (B side) to the high-concentration side (A side) across the membrane against the concentration gradient between both sides. In this transport form, the material, S, is transported according to the concentration gradient of the complex, CS, in the membrane. This uphill transport of S is attributed to the conjugated energy for the transport of the complex CS′ between the species S′ on the R side and the carrier C from the A side to the B side.

There are two types of uphill transport, as shown in Figure 34.16 (Uragami, 1992). Figure 34.16a is an antiport (countertransport) for the uphill transport of the species S and S′. Figure 34.16b is a symport (cotransport) for the uphill transport of the species S and S′. The uphill transport of species S in both cases requires conjugated energy due to the transport of species S′, since the amino groups in the chitosan molecules have a property as a basic group. These amino groups could be used as a fix career. Therefore, when the chitosan membrane is set up between an acidic solution and a basic solution, the amino groups could act as carriers for the uphill transport of anion species. The membranes prepared from mixtures of chitosan, PVA, and GA were insoluble in the acidic and the basic solution (Uragami et al. 1983a). These membranes were applied to the carrier transport for halogen ions (Uragami et al. 1983b). An example of the concentration change of Br^- ion and Na^+ ion and the pH changes in both the B side (basic side) and the A side (acidic side) with time due to the transport through the membrane is shown in Figure 34.17, where the membrane was prepared from the chitosan/PVA ratio of 40/60 (wt%), the L side was 0.1 M NaBr in 0.1 M NaOH, and the A side 0.1 M HBr.

The concentration of Br^- ion in the B side increased up to a maximum and then decreased with time. The concentration changes of Br^- ion in both sides were in the opposite direction. The increase of the Br^- ion concentration in the B side suggests that Br^- ions were actively transported across the membrane from the A side to the B side against its concentration gradient between both sides of the membrane because the initial concentration of Br^- ion was originally identical in both sides. The pH in the A side and the L side kept acidic and basic, respectively, for a long time. This result is attributed to the fact that the initial concentration of the OH^- ion in the L side is equal to that of the H^+ ion in the A side. The Na^+ ion concentration in the B side decreased with time caused by the diffusion through the membrane on the basis of its concentration gradient between both sides of the membrane. Since the membrane used in this work is an anion exchange membrane, it should be difficult to transport Na^+ ions across the membrane. However, Na^+ ions were transported from the B side to the A side across the membrane. This is due to the fact that the membrane is relatively open. In other words, this result also contains a possible mechanism that would counterdiffuse Br^- ions

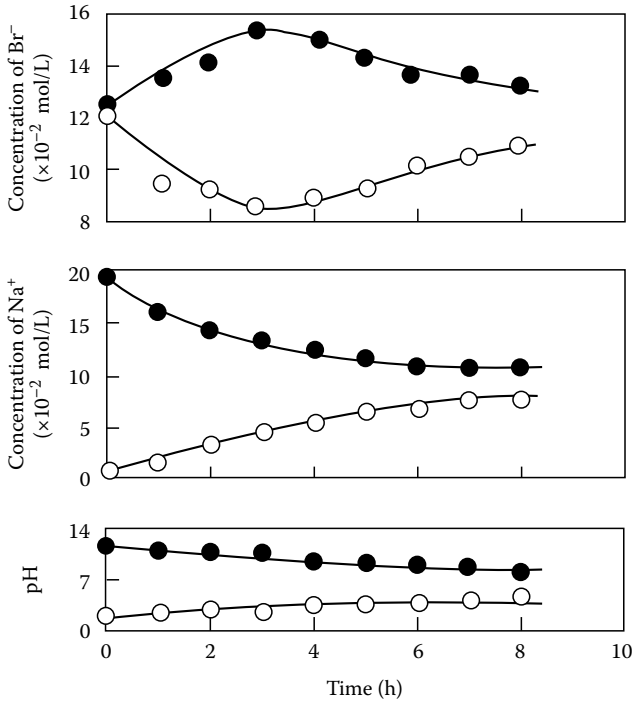


FIGURE 34.17 Changes of the Br^- and the Na^+ ion concentrations and pH with time on both sides through the membrane from the chitosan/PVA ratio of 40/60. L side: 0.1 M NaBr and 0.1 M NaOH; R side: 0.1 M HBr. (From Uragami, T. et al., *J. Appl. Polym. Sci*., 28, 136, 1983. With permission.)

in the B side transported actively across the membrane. Also, in the system of the Cl^- ion and the I^- ion, similar results were obtained. It is expected that the transport of halogen ions in such system, where one side was acidic and the other basic, is significantly influenced by a pH difference between both sides and the diffusion of the counter cation.

The transport fraction and the transport rate of Br^- ion are calculated from Equations 34.2 and 34.3, respectively.

$$\text{Transport fraction } (\%) = \frac{[X^-]_{max} - [X^-]_0}{[X^-]_0} \times 100 \tag{34.2}$$

$$\text{Transport rate (mol/L h cm}^2) = \frac{[X^-]_{max} - [X^-]_0}{At_{max}} \tag{34.3}$$

where

$[X^-]_0$ and $[X^-]_{max}$ are the initial and the maximum concentrations of the halogen ion in the B side, respectively

A is the membrane area

t_{max} is the transport time for $[X^-]_{max}$

These results under such conditions are caused by the fact that the pH in the B side and the A side are kept basic and acidic, respectively, for a long time. When the initial pH in the B side was lower or higher than 13.0, the pH in both sides became rapidly acidic or basic with time. These pH changes are attributed to a transport of H^+, OH^-, and Na^+ ions, caused by a proton-jump mechanism, specific

diffusion mechanism, and diffusive transport, respectively, as well as the transport of the Br^- ion caused by the uphill transport, in both sides. Consequently, both the transport fraction and the transport rate of the Br^- ion were smaller than those at pH 13.0. The permeation fraction of the Na^+ ion, determined by Equation 34.4, from the B side to the A side through the membrane increased as the Na^+ ion concentration in the B side increased.

$$\text{Permeation fraction } (\%) = \frac{[Na^+]_{A,t}}{[Na^+]_{B,0}} \times 100 \tag{34.4}$$

where

$[Na^+]_{B,0}$ is the initial concentration of the Na^+ ion in the B side

$[Na]_{A,t}$ is the Na^+ ion concentration in the A side after t hours when the Br^- ion concentration in the B side is maximum

A tentative mechanism of the uphill transport of halogen ions is shown in Figure 34.18.

In order to simplify the explanation, we instance the transport of the Br^- ion. When the Br^- ion is incorporated into the membrane on the A side (H^+ side), the hydrobromide is formed in the presence of hydrobromic acid and transferred through the membrane. As this hydrobromide reaches the B side (OH^- side), the hydrobromide changes to the amino group by neutralization and the Br^- ion is released. The released Br^- ion is transferred to the B side by the electric potential gradient between both sides. Consequently, it was suggested that the Br^- ion is actively transported through the membrane from the acidic side to the basic side.

If a greater pH difference and an electric potential difference between both sides could be kept for a long time, that is, the diffusive transport of metal ion from the B side to the A side should be prevented, the uphill transport of Br^- ion might be promoted. This expectation has been revealed by the result in Figure 34.19, in which the uphill transport of the Cl^- ion through the chitosan membrane was promoted by trapping the metal ions with crown ether in the basic side in order to prevent the diffusion of metal ion from the basic side to the acidic side.

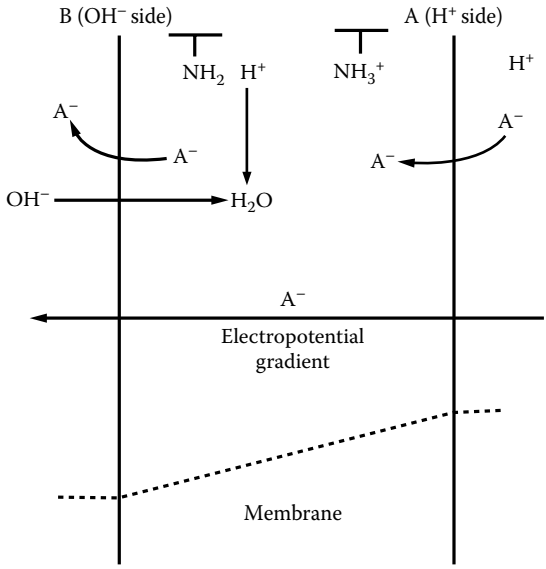


FIGURE 34.18 Tentative mechanism the uphill transport of halogen ions or organic anions through the chitosan membrane. (A^-) halogen ion or organic anion: (M^+) metal ion.

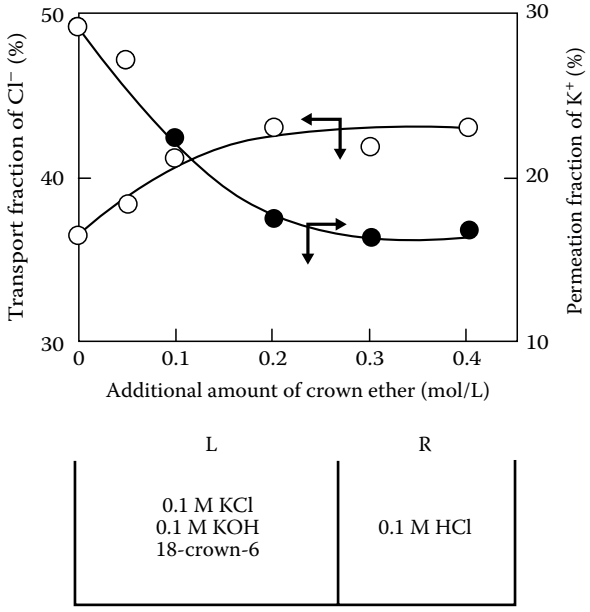


FIGURE 34.19 Facilitation of the uphill transport of Cl^- ion through the chitosan membrane by adding of 18-crown-6 in the basic side.

It was reported that an efficiency of the uphill transport of metal ions through cation exchange membranes was enhanced by an occurrence of a highly electrochemical potential gradient in the membrane (Uragami et al. 1983c; Wada et al. 1985). Therefore, the facilitation of the uphill transport of Cl^- ions in Figure 34.19 is attributed to an increase of the electrochemical potential gradient in the chitosan membrane based on a prevention of the diffusion permeation of K^+ ions with the addition of 18-crown-6 (Uragami et al. 1982). The chitosan membranes could actively transport benzoate ion and benzene sulfonate ion from the acidic side to the basic side against their concentration gradients as well as halogen ions (Uragami et al. 1982), and also *L*-phenyl alanine from the basic side to the acidic side against its concentration gradient (Uragami et al. 1991). The uphill transport is due to a tentative mechanism, as shown in Figure 34.20.

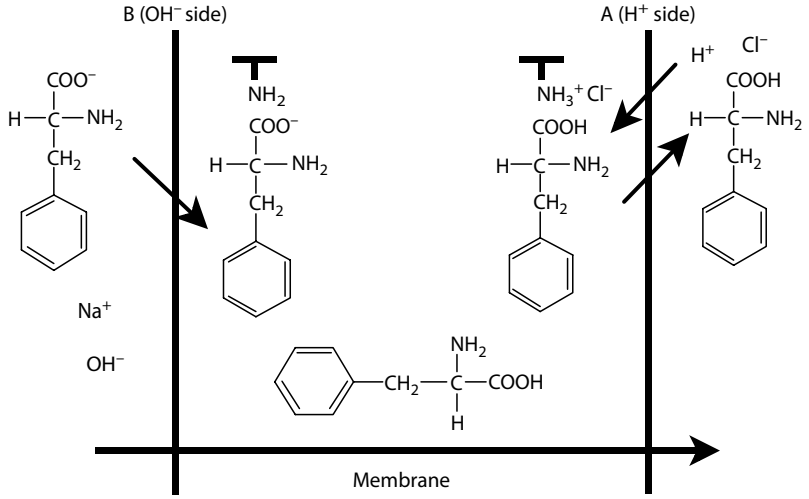


FIGURE 34.20 Tentative mechanism the uphill transport of amino acids through the chitosan membrane.

TABLE 34.3
Transport Direction of Uracil (Ura), Cytosine (Cyt), Adenine (Ade), Guanine (Gua), and K^+ Ion in the Transport against the Concentration Gradient of Them through the q-Chito Membrane

	Initial pH on B Side			
Transporting Species	**11.0**	**12.0**	**13.0**	**13.5**
K^+	B → A	B → A	B → A	B → A
Ura (9.5)	B ← A	B ← A	B ← A	B ← A
Cyt (4.5, 12.2)	B → A	B → A	B → A	B ← A
Ade (4.15, 9.8)	B → A	B → A	B → A B ← A	B ← A
Gua (3.2, 9.6, 12.4)	B → A	B → A	B → A B ← A	B ← A

Source: Uragami, T. et al., *Carbohydr. Polym.*, 21, 289, 1993.

Note: Arrows indicate the transport direction. Values in parentheses are the pK_a and pK_b value for nucleic acid bases. The initial pH on the A side was kept at 1.0 and the initial pH on the B side was changed.

A combination of the tentative mechanism in Figures 34.16 and 34.18 could suggest that if the solution containing a mixture of amino acid and organic acid is added on both sides across the chitosan membrane and if one side of the membrane is adjusted to acidic and the other to basic, an amino acid and an organic acid in the mixture could be actively transported against their concentration gradients through the chitosan membrane from the basic side to the acidic side and vice versa, respectively, and consequently the amino acid and the organic acid from their mixture could be separated or concentrated by a cross-selective transport. Nucleic acid bases such as adenine, guanine, uracil, and cytosine could also be actively transported against their concentration gradients through the chitosan membrane and the direction of transport for them was significantly dependent on the pH of both sides (Uragami et al. 1993). Quaternized chitosan membranes cross-linked with ethylenglycol diglycidylether were applied to the uphill transport of nucleic acid bases. The transport results are summarized in Table 34.3. Uracil was transported against its concentration gradient from the basic side to the acidic side regardless of the pH on the basic side. Cytosine, adenine, and guanine were also transported against their concentration gradients, but the direction of their transport depended upon the pH on the basic side. In particular, transport directions for adenine and guanine were switched during identical transport experiments.

34.3 CONCLUSIONS

In this chapter, the structure and the characteristics of various functional membranes such as gas permeation, dialysis, reverse osmosis, ultrafiltration, pervaporation, evapomeation, carrier transport membranes from chitin, and chitosan derivatives were described.

Recently, the utilization of biomass to various fields has been highly noted. To select chitin and chitosan derivatives, which can be naturally produced and easily got, as the membrane materials contributes to the utilization of biomass as the resources, and in addition conform to the social request such as the energy saving and the conservation of environment. Chitin and chitosan have many functional groups, and can be easily introduced hydrophilic and hydrophobic groups. Since modifications to the membrane materials agreed to are possible, we hope that excellent membranes from chitin and chitosan appear in the near future.

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35 Hydrophobically Modified Acylated Chitosan Particles for Drug Delivery Applications: An Overview

R. Shelma and Chandra P. Sharma

CONTENTS

35.1 INTRODUCTION

Interest in natural biopolymers for diversified applications in life is increasing because of its favorable properties such as availability from replenishable agricultural or marine food resources, biocompatibility, and biodegradability. Chitosan a natural polysaccharide derived from crustacean shell waste is of great importance because of its ecological safety and the possibility of preparing chemically modified derivaties for specific end uses. It consists mainly of β(1–4)-2-amino-2-deoxy-D-glucopyranose and a small amount of *N*-acetyl-D-glucosamine (Figure 35.1). Chitosan can be obtained by the deacetylation of chitin, which is the constituent component of the exoskeleton of arthropod, crustacean shells, and cuticles of insects (Muzzarelli 1977). Chitin ranks second to cellulose as the most plentiful organic compound on earth.

Chitosan is generally considered to have a degree of acetylation up to 60%. This degree of acetylation depends on the processing conditions of chitosan and its molar mass (Domard and Domard 2002). The sugar backbone consists of β(1–4)-linked D-glucosamine with a degree of *N*-acetylation. The structure of chitosan is very similar to cellulose; the only exception is that the amino group replaces the hydroxyl group on the C-2 position of cellulose. The structural variations of chitosan with cellulose are shown in Figure 35.2. Chitosan has a rigid crystalline structure through inter- and intramolecular hydrogen bonding.

35.2 METHODOLOGY FOR CHITOSAN PREPARATION

Chitosan is usually prepared by the deacetylation of chitin. The conditions used for deacetylation will determine the molecular weight and the degree of deacetylation. Chitin is found in the exoskeleton of some arthropods, insects, and fungi. Commercial sources of chitin are the shell wastes of crab, shrimp, lobster, etc. Proteins present in shrimp or crab shells are removed by alkaline treatment with 3%–5% NaOH (w/v) aqueous solution at room temperature overnight. Other inorganic constituents that remain in the products are removed by alkaline treatment with [3%–5% NaOH (w/v)]

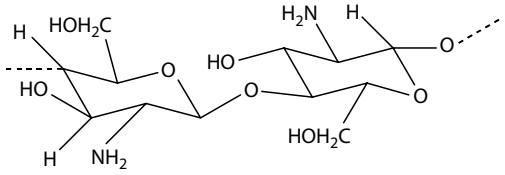


FIGURE 35.1 Chitosan.

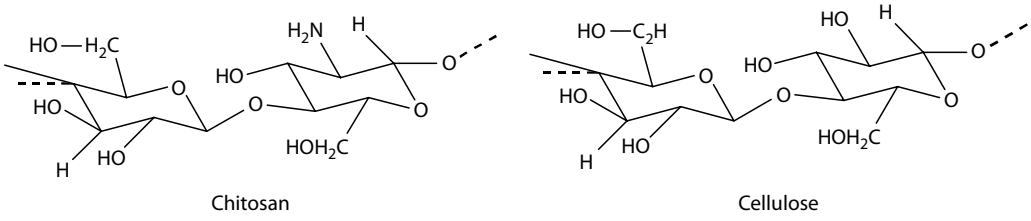


FIGURE 35.2 Structures of chitosan and cellulose.

aqueous solution at room temperature for 5 h. The product is reacted again with 40%–45% NaOH solution at 120°C for 4–5 h giving the crude sample of chitosan. The crude sample is then purified by precipitating the chitosan from its aqueous acetic acid solution to NaOH and washed with distilled water until it is neutralized (Hirano 1996).

Chitosan has been widely used for many biomedical applications because of its favorable characteristics such as biodegradability (Struszczyk et al. 1991), biocompatibility (Chandy and Sharma 1990, Hirano et al. 1990), inexpensiveness, and nontoxicity. However, the level of toxicity of the chitosan depends on the molecular weight and the degree of deacetylation. Hirano and Knapezk et al. has reported that chitosan has low oral toxicity with an LD_{50} in rats of 16 g/kg, which is close to that of salt or sugar (Hirano et al. 1990, Knapezk et al. 1984). Chitosan is also bioactivite and bioadhesive compared with other natural polymers commonly used in drug delivery. It has antacid and antiulcer properties that prevent or weaken drug irritation in the stomach (Kumar 2000).

The major drawback of chitosan is its poor solubility at physiological pH. It is soluble only at aqueous acidic solutions having a pH < 6.5. Hence, chitosan needs to be modified to enhance its solubility. The presence of reactive amino group and hydroxyl groups (one primary hydroxyl group at C-6 and one secondary hydroxyl group at C-3) would be highly beneficial for chemical modification and can be modified without disturbing the degree of polymerization. However, the reaction should be performed under homogenous conditions; the chemical reaction may otherwise run into a number of problems like poor extent of reaction, difficulty in regioselective substitution, structurally nonuniform products, and partial degradation due to severe reaction conditions. The covalent modification of chitosan and other polysaccharides to produce material for specific applications is currently an interesting area of development. Chemically modified chitosans have a wide range of pharmaceutical applications, and modification is a powerful tool for controlling the interaction between polymer and drug and enhancing the loading capacity, as well as controlling the drug release from the matrix. Modified chitosan improves the bulk properties for sustained release.

The most extensively studied modification of chitosan is acylation. The product is an amide because amides are more stable molecules compared to acyl carbonyls. This is due to the resonance stabilization of the lone pair of electrons on nitrogen to the carbonyl pi system (Figure 35.3).

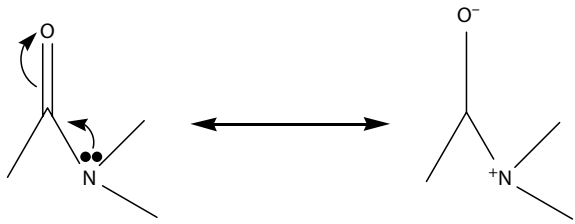


FIGURE 35.3 Resonance stabilization of amide.

N-Acylation can be achieved with various fatty acid anhydrides. Long chain fatty acid modification of polymers enhances their solubility due to their hydrophobic backbone and hydrophilic end groups. Long chain fatty acyl derivatives of chitosan are an interesting hydrophobic modification method that is used to form nanoparticles. Fatty acids are carboxylic acids with long hydrocarbon chains. The hydrocarbon chain length may vary from 10 to 30 carbons. The nonpolar hydrocarbon alkane chain is an important counterbalance to the polar acid functional group. In acids with only a few carbons, the acid functional group dominates and gives the whole molecule a polar character. But in fatty acids, the nonpolar hydrocarbon chain gives the molecule a nonpolar character. There are two groups of fatty acids: saturated and unsaturated. Hilano et al. reported that the solubility of chitosan can influence the acyl chain length with which acylated and the degree of substitution. Derivatives with a short chain length (up to C_8) with a low-to-moderate degree of substitution exhibit solubility in water but with a higher degree of substitution display very little or no solubility in water. Because of the increase of hydrophobicity, acyl chitosan derivatives with longer chain lengths are insoluble in water regardless of the degree of substitution.

Various acylation reactions of chitosan can take place. The acylation of chitosan can take place using either acyl chloride or anhydride. The principal mediums used for acylation are aqueous acetic acid/methanol, pyridine, pyridine/chloroform, trichloroacetic acid/dichloroethane, ethanol/methanol mixture, methanol/formamide, or dimethylacetamide-LiCl (Shigemasa et al. 1999). Chitosan is a multinucleophilic polymer; due to the presence of *N*-amino and hydroxyl group, acylation can take place at both groups and forms either amide or ester. The initial site at which acylation occurs is the amino group, which is more nucleophilic than the hydroxyl group. But *O*-acylation can take place by changing the experimental condition and by protecting the amino group. We can get either *N*-acyl (Hirano et al. 2002, Seo et al. 1989, Tien et al. 2003) or *O*-acyl (Sashiwa et al. 2002), or both (Grant et al. 1990, Wu et al. 2005, Zong et al. 2000), by controlling the reaction conditions because of the fairly different reactivities of the two hydroxyl groups and the amino group of chitosan. These hydrophobic derivatives are more organic soluble than native chitosan. Hydrophobic chitosan derivatives with saturated and unsaturated acyl groups have been successfully prepared and the substituent is randomly distributed in a controlled amount along the chitosan chain.

Acylation can also be done by using cyclic anhydrides via ring-opening reaction (Sashiwa et al. 2000). Bulky and cyclic anhydrides exploit the ease with which the substituents reduce the regularity of chitosan, and the product is water soluble below pH 4 and above pH 7. This physical property is due to the pronation of amino groups to NH_3^+ in acidic medium (below pH 4) and change of carboxyl group to carboxylate ion in the basic region. But the insolubility between these ranges is due to the presence of equimolar NH_3^+ and COO^- groups at the isoelectric point. The reaction of chitosan with lactone results in hydroxyl-substituted carboxylchitosan, *N*-hydroxyalkanoyl groups (Loubaki et al. 1989).

The thermolysis of chitosan with acylammonium salts also results in the acylation of chitosan (Toffey and Glasser 2001, Vasnev et al. 2006). Release studies of drugs by using acyl derivatives with longer chains suggest that the release is controlled by either diffusion or by swelling followed by diffusion depending on both the acyl chain length and the degree of acylation (Tien et al. 2003). Various routes for *N*-acylation of chitosan have been shown in Figure 35.4.

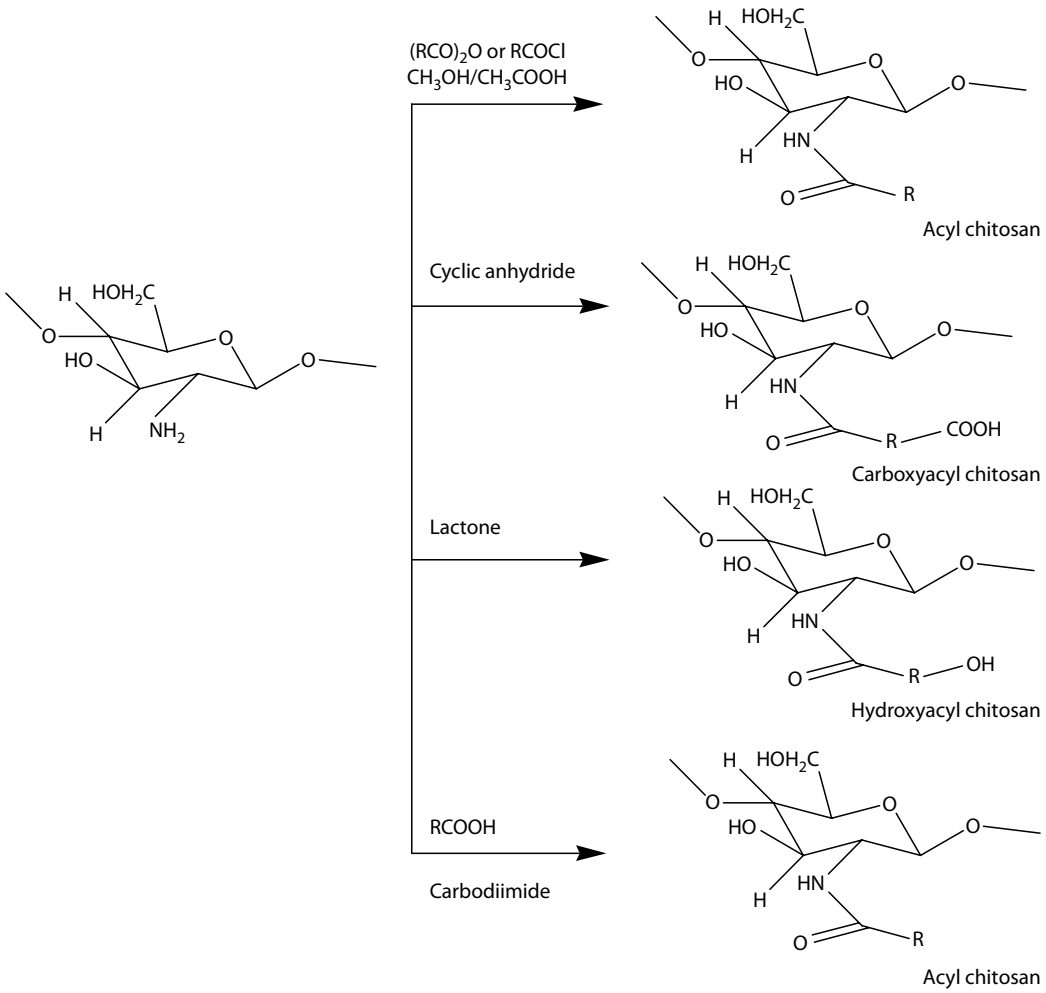


FIGURE 35.4 Routes of acylation.

We can completely synthesize regioselective *N*-acylated chitosan by protecting the hydroxyl group with the trityl group. For this, the amino group is first protected with the phthaloyl group to avoid tritylation at the amino group; after tritylation, the phthaloyl group is removed with hydrazine hydrate treatment. When acylation is completed, the trityl group is removed with the help of hydrochloric acid (Figure 35.4). It has been reported that *N*-chloroacyl-6-*O*-triphenyl methyl chitosan has been prepared by using this protection method (Holappa et al. 2004, 2005, 2006).

Kurita et al. (1988) have synthesized regioselective *N*-nonanoyl chitosan and reached a conclusion that acylation enhances the adsorption capacity, and the capacity increases from 75% of chitosan to 98% of nonanoyl chitosan.

We can also synthesize acylated chitosan at the hydroxyl group. Thus, the formed derivative has two main benefits: the organic solubility due to the hydrophobic group and the presence of ester linkage that can hydrolyze by the enzyme-like lipase. Hence, most of the *O*-acyl chitosan derivatives are biodegradable. Selective *O*-acylation can be achieved by using methane sulfonic acid as a solvent. This happens due to the salt formation of the amino group with methane sulfonic acid. However, the protecting effect of methane sulfonic acid on the amino group is not clear yet (Figure 35.5). Another route for *O*-acylation is by protecting the amino group. This procedure involves the protection of the amino group by the phthaloyl group, acylation at the hydroxyl group, and, finally, the

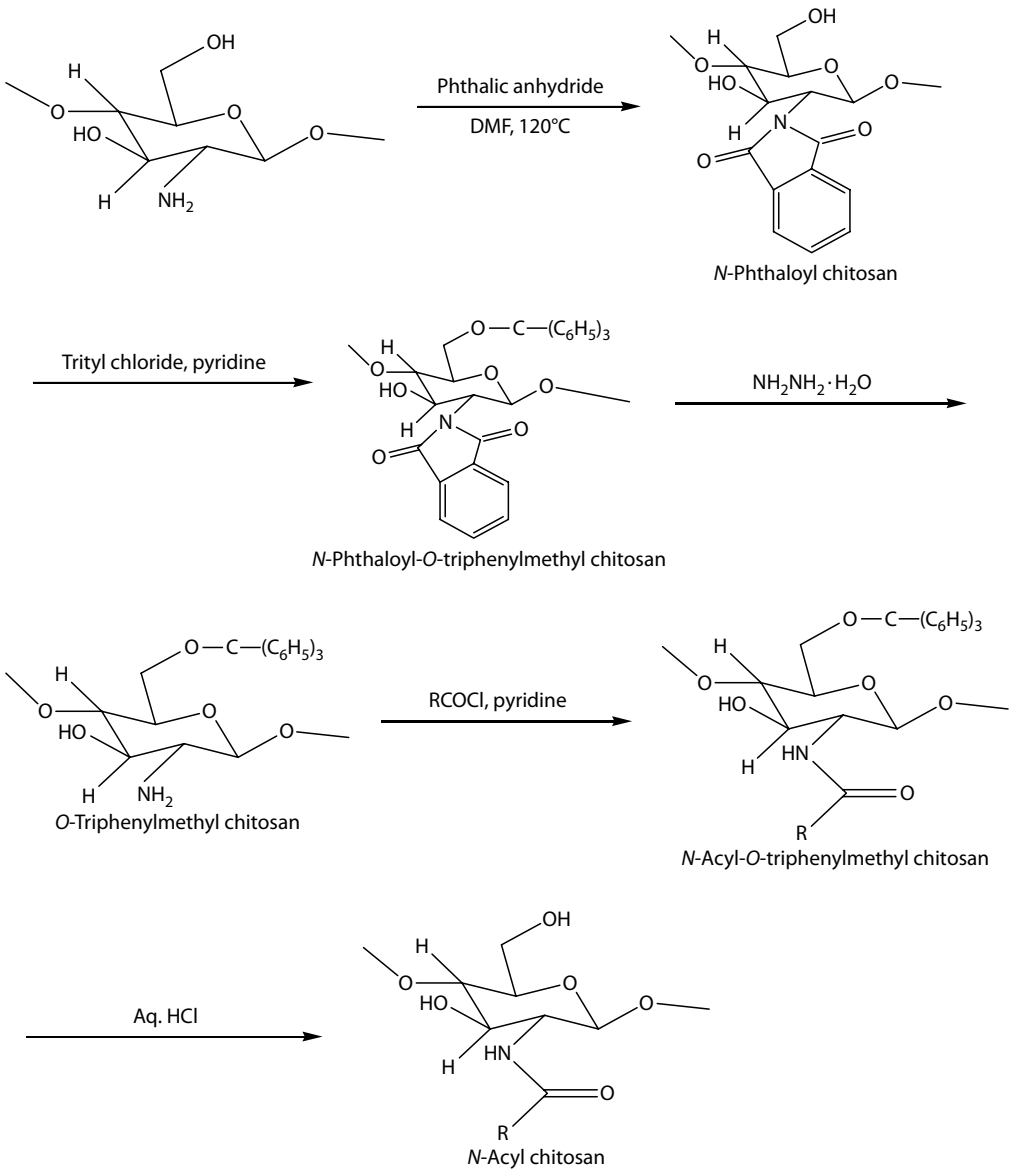


FIGURE 35.5 Regeoselective *N*-acylation.

removal of the protecting group by using hydrazine hydrate. However, this procedure requires only a single step instead of multi steps as mentioned in the case of earlier procedures (Figure 35.6).

Acylation of chitosan can be confirmed by using FTIR, NMR, etc., comparing with native chitosan. The degree of substitution can also be determined from IR spectra. The degree of acylation can be determined by the ninhydrin test and by elemental analysis.

Since this chapter is focused mainly on the hydrophobic chitosan, *N*-acylation of chitosan with various fatty acid chlorides with a hydrophobic backbone is utilized to increase its hydrophobic character and to develop important changes in its structural features (Tien et al. 2003). Tien et al. have reported that acylation with fatty acid with carbon atoms C_6–C_{16} increases the hydrophobicity, and these derivatives are applied for drug delivery (Tien et al. 2003). Reports have shown that chitosan with a higher degree of deacetylation was more susceptible to acylation. It is also reported

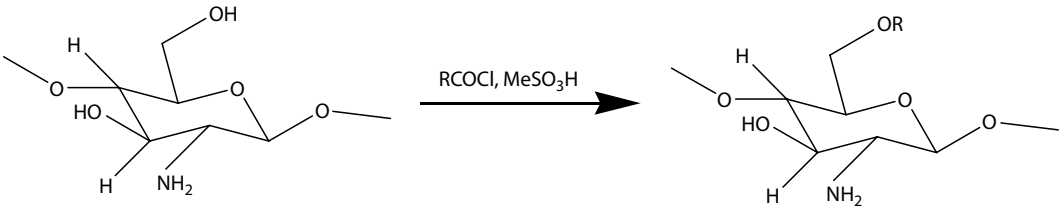


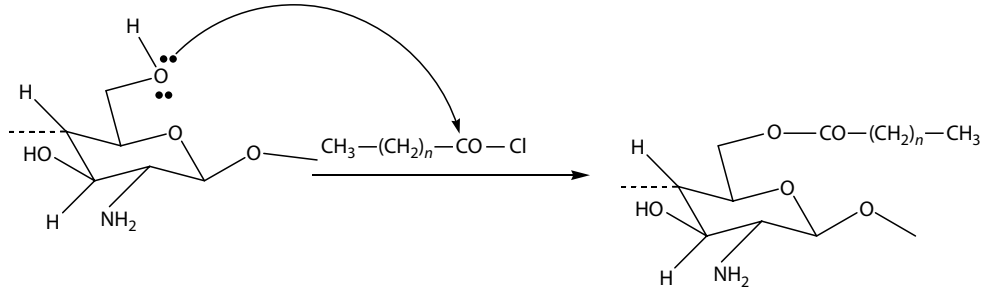
FIGURE 35.6 *O*-acylation.

that the acylated chitosan is blood compatible, and blood compatibility of acylated chitosan can be explained on the basis of the balance in the hydrophobic long acyl group and hydrophilic chitosan. Hirano et al. (1985) found that the hydrophilic nature was altered by the hydrophobic group, and a balance in the hydrophobic and hydrophilic properties improved the blood compatibility. Lee et al. (1995) also investigated the blood compatibility of acyl chitosan films using rheological methods.

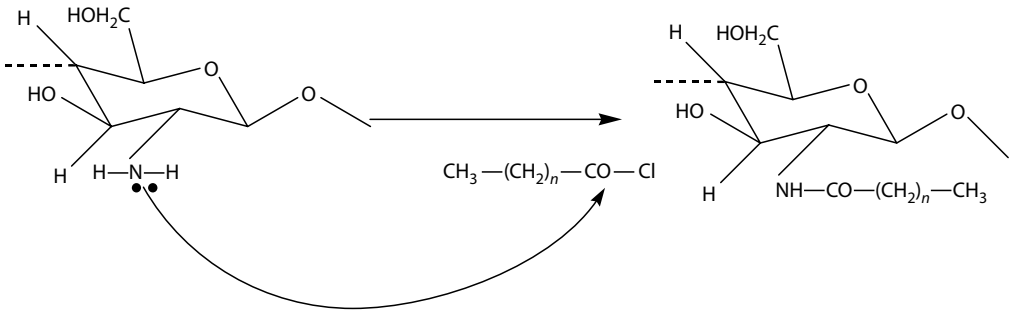
Acylation with fatty acid chlorides can take place at either the amino group or the hydroxyl group. The mechanism of the reactions is shown in Schemes 35.1 and 35.2. In Scheme 35.1, the reaction takes place at the hydroxyl group. Here, the nucleophilic reaction is that of the hydroxyl group of chitosan on the carbonyl group of acid chloride. The primary hydroxyl group at C-6 position is most sterically accessible and more reactive. However, the secondary hydroxyl group at C-3 position also seems to participate in the reaction but at very low percentage.

In Scheme 35.2, acylation proceeds through the amino group of chitosan. Here, the nucleophilic reaction is that of the amino group on the carbonyl group of acyl chloride. The nucleophiles have a couple of free electrons, hence the reaction is like that of a Lewis base.

Acylated chitosan would reduce the hydration of the matrix and the network is stabilized because of its hydrophobic interaction. Acyl chitosan with longer acyl chain length (C_{16}) remains



SCHEME 35.1 Acylation at OH group.



SCHEME 35.2 Acylation at NH_2 group.

unchanged in aqueous media due to strong hydrophobic interaction but reverse effects are observed with short chain length. For medium size (C_8–C_{14}) chain length, the effect is in between that for long and short chain length.

Tien et al. (2003) synthesized *N*-acylation using various fatty acids like palmitoyl chloride, caproyl chloride, octanoyl chloride, and myristoyl chloride, and studied the release of acetaminophen. Mechanical properties like crushing strength were determined by compression testing. Chitosan has a weak tablet crushing strength, and acylated chitosan with chain length C_6 also has weak strength but exhibited significant swelling. However, acylated chitosan with higher chain length showed lower swelling but higher crushing strength. Crushing strength is therefore related to the length of acyl chain. Crushing strength increases with an increase in the length of the chain, which is due to the hydrophobic interaction. The release of acetaminophen from acyl chitosan matrices has also been done using spectrophotometer. The release of drug from oleoyl and myristoyl chitosan is for a very short time but that of caproyl chitosan is moderately long. Longer drug release is obtained for palmitoyl chitosan. When hydrophobicity increases, the matrix limits the access of water and the dissolution of drug. The high stability of the matrix is due to the hydrophobic interaction. However, caproyl chitosan shows better mechanical properties than octanoyl and myristoyl chitosan. Because of the longer release and stability of the matrix, palmitoyl chitosan is usually a chosen matrix for the drug compared to caproyl, octanoyl, and myristoyl chitosan.

Liu et al. (2005) have prepared hydrophobic chitosan nanoparticles with linoleic acid for protein delivery. Linoleic acid is an essential fatty acid that exists as a positional and stereoisomer of octadecadienoic acid, and this type of polyunsaturated fatty acid can sensitize tumour cells to chemotherapy and radiotherapy. This has been proved in cell culture, tumour-bearing animals, and, finally, in humans (Conklin 2002, Germain et al. 1998, Vartak et al. 1997). Linoleic acid plays a major role in fatty acid metabolism in the human body. Liu et al. have developed biocompatible amphiphilic lenoleic acid chitosan nanoparticles (100–500 nm), which can be used for protein delivery applications. The loading efficiency decreases with increasing concentration of Bovine Serum Albumin (BSA), and the nanoparticles are saturated with BSA that has a concentration of 0.5 mg/mL and a loading capacity of 37.57% ± 0.25%. BSA forms complexes with a derivative that has a hydrophilic chitosan backbone and a hydrophobic domain of linoleic group.

DNA can deliver with acylated chitosan with deoxycholic acid and 5β-cholanic acid due to its high transfection efficiency because of an increase in the cell–membrane carrier interaction and destabilization of cell membrane (Kim et al. 2001, Yoo et al. 2005). Bhattarai et al. (2008) prepared biocompatible hydrophobic chitosan-gold nanoparticles having the size 12 nm by grafting acylated chitosan like oleoyl, caproyl chitosan on gold nanoparticles for DNA delivery and found them to be noncytotoxic at low concentrations. The aqueous solution of *N*-acyl chitosan-gold nanoparticles can bind DNA and form complexes through electrostatic interaction; because of its favorable characteristics like positive zeta potential and nanosize, the matrix will be a more attractive vector for gene delivery, especially oral gene therapy.

Naberezhnykh et al. prepared hydrophobic chitosan and studied their interaction with lipopolysaccharide (LPS) of gram-negative bacteria (Naberezhnykh et al. 2008). The degree of *N*-acetyl group also plays a significant role in the interaction of LPS with chitosan. The interaction decreases with an increase in acyl group because of the increase in crystallinity and the decrease of charge density. It has been studied that the size and localization of chitosan-binding sites on LPS molecules depends on linear charge density and flexibility of chitosan when studying the interaction between polyelectrolyte and proteins (Kayitmazer et al. 2003). It has also been reported that acylated polyamines having carbon atoms 14–16 in the acyl chain are more effective binders for LPS (David et al. 1999).

One of the interesting features of fatty acid is the permeation enhancement. Palin et al. (1986) studied the absorption enhancement of various fatty acids on cefoxitin absorption in rat intestinal loops in situ. They ranked the fatty acids enhancement property as lauric acid > palmitic acid > caprylic acid > oleic acid. But in the study of in situ colonic cefmetazole absorption in rat and of

in vitro colonic permeation of inulin, they all reached at a conclusion that sodium caprate is the effective enhancer compared to sodium laurate or sodium oleate (Tomita et al. 1988). Oleic acid is a better enhancer for intraduodenally administered carboxyfluorescein absorption in rat at 16 and 32 mM but it has no effect for 8 mM. Intracolonic administration showed better absorption enhancement than intraduodenal administration (Murakami et al. 1986). It has also been reported that the absorption enhancement for 50 mM fatty acids in the order caprate > laurate > oleate. But here caprate and caprylate showed more enhancing effect after colonic administration than duodenal administration (Muranushi et al. 1993).

Fatty acid–modified chitosan derivatives enhance the passage of drugs through the paracellular route, which is difficult for that drug when given directly. The bioavailability of some drugs reduces due to its poor paracellular transport. But medium chain fatty acids have been shown to have the enhancing property across the passage through the paracellular route. Sodium caprate, a medium chain fatty acid, has been widely used as an effective enhancer in clinical setting and it has been proved that it has no side effects (Lindmark et al. 1998, Sakai et al. 1997). To clarify this, Lindmark et al. (1995) checked the enhancing effect of fatty acids like sodium caprylate (C_8), sodium caprate (C_{10}), and sodium laurate (C_{12}). They also did a comparative study with sodium caproate and reached at a conclusion that C_8, C_{10}, and C_{12} exhibit dose-dependent enhancing effects on mannitol transport across the cell monolayer. Among the three, C_{12} was the most effective enhancer.

The mechanism of paracellular transport enhancement by sodium caprate was via phospholipase C activation and upregulation of intracellular Ca^{2+}, leading to the contraction of calmodulin-dependent actin–myosin filaments and opening of tight junction. Because of the low molecular weights of fatty acids, it can easily absorb from the intestine than the drug itself.

Cogan and Garti have found that the enhancement action of fatty acids in dermal and transdermal drug delivery is due to the penetration of the fatty acid moiety into the lipid bilayers of stratum corneum (Cogan and Garti 2006).

Nowadays, polymeric micelles have been given attention as novel colloidal delivery systems because they can fulfill the requirements of an ideal and versatile drug carrier (Aliabadi and Lavasanifar 2006). They can self-assemble in an aqueous environment and possess a core shell structure. Hydrophobic drugs, proteins, DNA, etc., can be encapsulated in the hydrophobic core and the hydrophilic shell interfaces the biological medium. They can be successfully used as a passive targeting carrier of anticancer drugs because they are structurally strong and are not captured by the reticuloendothelial cell system. However, chitosan cannot form micelle but its derivative with both hydrophobic acyl groups and hydrophilic groups could form micelle and solubilize hydrophobic drug (Yoshioka et al. 1995).

Miwa et al. synthesized *N*-lauroyl-*O*-carboxymethyl chitosan for anticancer drug, taxol carrier. This derivative can solubilize taxol by forming micelles having a particle size less than 100 nm and is considered as a very good matrix for anticancer drug, taxol (Miwa et al. 1998). Polymeric micelles from amphiphilic copolymers can be used for poorly soluble drugs and can enhance the water solubility and bioavailability of various poorly soluble drugs. Chen et al. (2008) have prepared *O*-acyl-*N*-trimethyl chitosan for the hydrophobic drug cyclosporine A and studied the effect of molecular weight, the degree of methylation, and the length of acyl chain in the drug-loading efficiency. From the studies, they have reached at a conclusion that chitosan derivatives from high-molecular-weight chitosan, medium-sized acyl chain length, and high degree of methylation would be the most promising carriers for poorly soluble drug. High-methylated derivatives showed greater drug loading. With a similar degree of methylation, derivative one which has higher molecular weight showed a better loading efficiency. High molecular weight derivate showed a better loading efficiency than low molecular weight derivative with the similar degree of methylation.

In 1987, Hirano et al. prepared sulfated *N*-fatty acyl chitosan for improving the solubility of acylated chitosan. Derivatives with acyl chain length less than C_{14} were found to be soluble in water but that with palmitoyl group was slightly soluble due to the hydrophobic property of the acyl group.

Rekha and Sharma (2009) synthesized the chitosan derivative with both hydrophilic (succinyl group) and hydrophobic group (lauroyl group) for enhancing gastrointestinal absorption of insulin and studied its mucoadhesivity and absorptivity. Insulin release studies are done in gastric as well as in intestinal pH. The loading efficiency obtained was 48 IU insulin/mg. The mucoadhesion of the derivative was 94% but that of native chitosan was 70% due to the interaction of lauroyl group with the hydrophobic domain of mucus. The insulin release is slow but sustained at gastric pH, which cannot be attained from native chitosan matrix. Moreover, its calcium-binding capacity helps the insulin from the enzymatic degradation (Rekha and Sharma 2009).

Our group synthesized self-assembled anacardoylated chitosan having a particle size of around 214 nm by the reaction of chitosan with anacardic acid, a natural fatty acid having bulky aromatic group as well as long aliphatic chain, and studied the release of insulin. The hydrophobic nature of the nanoparticles help in the sustained release of insulin in the intestinal environment, and the released insulin was stable and retained its conformation. Since anacardic acid is a natural fatty acid and an aspirin derivative, these nanoparticles may be translocated efficiently across the intestinal epithelium and may also be biocompatible (Shelma and Sharma 2009).

35.3 CONCLUSION

Chitosan is widely used for pharmaceutical applications. However, as chitosan does not dissolve in neutral and basic aqueous media, its use is limited. Chemical modifications of chitosan, however, provide derivatives that are soluble at neutral and basic pH. Moreover, chemical modifications can be used to attach various functional groups and to control hydrophobic and cationic properties. Chitosan derivatives exhibit the very properties that have the potential to be used in a wide range of biomedical applications. The delivery of drugs and interactions with living tissues seem to be major topics in the current research on chitosan. This chapter focuses on the various acylated chitosans for drug delivery applications. Hydrophobic self-assembly can enhance the stability of the hydrophobic matrix, and the release of the drug is controlled either by diffusion or swelling followed by the diffusion depending on the degree of acylation and the chain length of the acyl group. A good drug delivery system is a system that can swell rapidly and is able to retain water in its swollen state without dissolution. In that sense, hydrophobic acylated chitosan holds much promise as a drug delivery systems. From various reports, much progress has been seen in the area of hydrophobic chitosan derivatives and this implies an unlimited possibility of applications. Further, basic and application-oriented studies are expected to fully utilize the potential of chitosan for advanced uses for the benefit of society.

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36 Chitin, Chitosan, and Their Derivatives in Beverage Industry

Aurélie Bornet and Pierre-Louis Teissedre

CONTENTS

36.1 INTRODUCTION

Chitin, chitosan, and their derivatives have been of interest in the past few decades due to their potential broad range of industrial applications. They already have commercial applications in the cosmetic industry, in the field of medical devices, and as food supplements (Hadwiger and Riggleman 1984; Lang and Clausen 1988; Ormrod and Miller 1998; Kittur et al. 2001). Besides their use as the aforementioned supplements, chitinous polymers may have unique applications in the food industry. Research and development in this area can be associated with numerous applications of these biopolymers, especially for the conversion of processing discards into valuable by-products and alternative specialty materials, which has been identified as a timely challenge for the food industry. Several studies have shown that chitin and chitosan, already value-added products from the mushroom and crustacean waste, may be of use in bioconversion for the production of value-added food products (Carroad and Tom 1978; Revah-Moiseev and Carroad 1981; Shahidi and Synowiecki 1991), the preservation of foods from microbial deterioration (Papineau et al. 1991; El Gaouth et al. 1992; Sudharshan et al. 1992; Fang et al. 1994; Chen et al. 1998), the formation of biodegradable films (Wong et al. 1992; Uragami et al. 1994; Butler et al. 1996; Chen and Hwa 1996; Hoagland and Parris 1996; Kittur et al. 1998), the recovery of waste material from food processing discards (Bough 1975, 1976; Bough and Landes 1976; Latlief and Knorr 1983; Senstad and

Mattiasson 1989; Hwang and Damodaran 1995; Sun and Payne 1996; Pinotti et al. 1997), and the purification of water (Jeauniaux 1986; Micera et al. 1986; Muzzarelli et al. 1989; Deans and Dixon 1992; Peters 1995; No and Meyers 2000; Guibal 2004; Crini 2005, 2006; Krajewska 2005; Gerente et al. 2007). The interest of using chitinous compounds in oenology (Bornet and Teissedre 2005) and for the clarification and deacidification of fruit juices has been lengthily documented (Imeri and Knorr 1988; Soto-perlata 1989; Knorr 1991; Zhang et al. 1991; Chen and Hwa 1996; Rwan and Wu 1996; Spagna et al. 1996; Freepson 1997; Shahidi et al. 1999; Goycoolea et al. 2000; Ravi-Kumar 2000; Struszczyk 2002; Agullo et al. 2003; Shalaby et al. 2003; Tripathi and Dubey 2004; Kosaraju 2005). Although research on chitin–glucan is less extensive, it has been recently launched in the market for applications in the wine industry.

36.2 CHITIN, CHITIN–GLUCAN, AND CHITOSAN

36.2.1 Chitin

Chitin is the most abundant natural biopolymer after cellulose. Chitin is a common constituent of exoskeletons of crustaceans and cell walls of fungi and insects (Bartnicki-Garcia 1968), composed of *N*-acetyl-D-glucosamine residues, as illustrated in Figure 36.1.

36.2.2 Chitosan

Chitosan is a linear polysaccharide composed of two repeating units (D-glucosamine units [GlcN] and *N*-acetyl-D-glucosamine [GLcNAc] units) randomly distributed along the polymer chain and linked by β(1-4)-bonds. Chitosan is produced by the chemical deacetylation (alkali treatment) of chitin, whose main industrial source is shellfish waste from food processing (shrimp, squid, and crab). The production of fungal chitosan has been more recently acheived by KitoZyme (BE). The chemical structure of chitosan is illustrated in Figure 36.2.

Chitosan is a polyamine with a high degree of positive charge at pH below pK_a of the amine groups (pK_a: ~5.5–6.5). Therefore, chitosan is prone to interact readily with negatively charged substances such as proteins, anionic polysaccharides, or fatty acids.

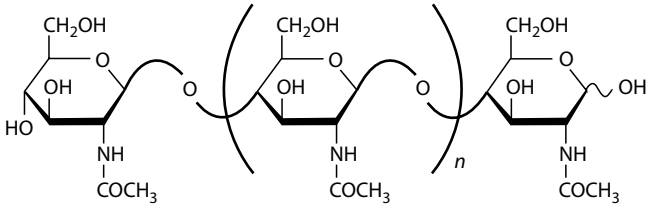

FIGURE 36.1 Chemical structure of chitin.

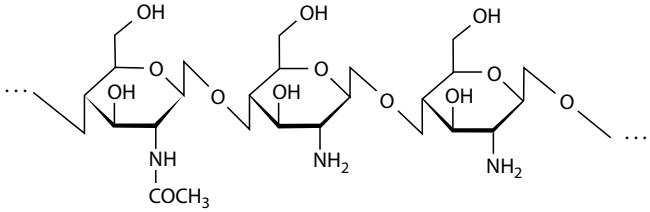

FIGURE 36.2 Chemical structure of chitosan.

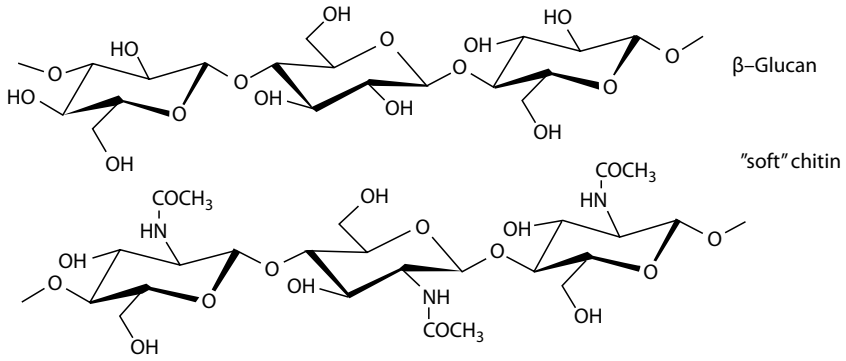

FIGURE 36.3 Chemical structure of chitin–glucan.

36.2.3 Chitin–Glucan

Chitin–glucan is a natural biopolymer comprising two types of polysaccharide chains, chitin (repeating units *N*-acetyl-D-glucosamine) and beta(1,3)-D-glucan (repeating units D-glucose), organized into a three-dimensional network.

Chitin–glucan is extracted from the mycelium of *Aspergillus niger*, which is a by-product of the production of citric acid for food and pharmaceutical industry. The chemical structure of chitin–glucan is illustrated in Figure 36.3.

36.3 APPLICATIONS IN WINE INDUSTRY

36.3.1 Clarification Agent

Hitherto, wine clarification products came from two basic sources—animal (gelatin, bovine albumin, isinglass, casein), or mineral (bentonite, silica gel). Gelatin has a long history of use as a clarifying agent. Gelatin derives from the collagen contained in animal skin, bones, and cartilage.

In Europe, the emergence of Creutzfeld–Jacob disease threw a new suspicion on animal-derived products used as adjutants in the food industry. Blood albumin is, for example, no longer authorized. Some suppliers also now exclusively require wines that have not been treated with animal proteins. Other recent crises in relation to food safety have caused several winemakers to consider alternative solutions for wine clarification. A consequence is the boost in the search for innovative nonanimal-derived products with equivalent efficiency compared to existing clarification agents and guaranteed nontoxicity (Lefebvre et al. 2000; Marchal 2000a,b, 2002a,b,c, 2003). From this point of view, the chitinous products derived from fungi appear attractive and promising alternatives to animal-derived product such as gelatin.

Indeed, chitosan has already been tested as a clarifying agent for beverages and most particularly for fruit juices. Chatterjee (2004) obtained a significant clarification effect by treating 50 mL of grape juice with 100 mg of soluble chitosan, an effect that was greater than the one obtained with either bentonite or gelatin. Furthermore, it was observed that this treatment had no impact on the chemical characteristics of the wine and that it enhanced sensorial qualities (appearance, color, taste). Another study on apple juice yielded similar results to the Chatterjee study (Soto-paralta 1989).

After different laboratory tests on small volumes, industrial trials using chitin–glucan in cellar has been performed by KitoZyme according to article 41 of EC regulation No. 1622/2000 for must settling and fining wines. In each trial, chitin–glucan was compared with fining agents traditionally used in wineries (gelatin, isinglass, casein, egg albumin). Results showed that in most of the cases, a technological effect equivalent or greater was obtained with chitin–glucan in comparison to the reference fining agent (Table 36.1).

TABLE 36.1
Effectiveness of Different Clarification Treatments on Chardonnay, Terret, and Sauvignon Musts

	Reduction (%) in Turbidity	
Grape Variety	**With Reference Treatment**	**With Chitin–Glucan Treatment**
Chardonnay	Pectolytic enzyme 1 (1 g/hL): 83%	Chitin–glucan (50 g/hL): 93%
Terret	Pectolytic enzyme 2 (2 g/hL): 93%	Chitin–glucan (70 g/hL): 93%
Sauvignon	PVPP (50 g/hL): 96%	Chitin–glucan (30 g/hL): 92%
	Bentonite (50 g/hL): 97%	Chitin–glucan (50 g/hL): 95%
	Gelatin (50 g/hL): 98%	
	Silica gel (25 ml/hL): 86%	
	Isinglass (2 g/hL): 98%	
	Casein (40 g/hL): 97%	

In addition to the technological performance of chitin–glucan, the lack of toxicity and allergenicity of this product has been clearly demonstrated.

36.3.2 Stabilization of White Wines

Maderization and browning adversely affecting wine color, taste, and aroma (Pallotta and Cantarelli 1979; Simpson 1982; Singleton et al. 1984; Piracci and Tamborra 1987) are problems often encountered in white wines. Among the different possible causes, the most important are the oxidation of the polyphenols including catechins, proanthocyanidins, hydroxycinnamic acids, and their derivatives and the formation of oligomers that cause browning. Spagna et al. (1996) demonstrated that chitosan has a good affinity to phenolic compounds, the main components responsible for browning of white wines. Spagna et al. (1996) demonstrated that chitosan has a good affinity to phenolic compounds, which are the main components involved in the wine oxidation processes responsible for browning. In particular, compared with conventional absorbents (potassium caseinate and polyvinylpolypyrolidone [PVPP]), the efficacy of chitosan treatments at the laboratory scale is lower on the flavin and proanthocyanidin classes and equal or greater on total polyphenols and hydrocinnamates, while the absorbance capacities of chitin is very low.

The possibility of regenerating chitosan for reuse in at least 14 cycles was also demonstrated (Spagna et al. 2000). The low price of chitosan and its possible reuse, with consequently reduced environmental impact, are all factors that make this biopolymer particularly interesting for industrial applications in winemaking as compared to caseinate.

36.3.3 Complexing Agent for Ochratoxin A

Ochratoxin A (OTA) is a known carcinogen nephrotoxin produced by several molds of the *Aspergillus Xavus* and *Penicillium* genera, including *Aspergillus ochraceus*. It has been frequently detected in foods, especially cereal products and beverages. In humans, exposure to OTA has been linked with Balkan endemic nephropathy, a chronic kidney disease associated with tumors of the renal system. Wine and grape juice has been identified as a possible source of ochratoxin.

The main molds responsible for OTA contaminations in grapes and wines are *Aspergillus carbonarius* and *Aspergillus nigri* (Bornet and Teissedre 2005; Jorgensen 2005; Bau et al. 2006; Leong et al. 2006a,b; Serra et al. 2006). The European Commission established with regulation 123/2005, allowed at 2 μg/L (ppb) the maximum concentration of OTA in wine, must, and grape juice.

A variety of other fining agents, including activated carbon, silica gel, potassium caseinate, egg albumin, and gelatin, have been used for their abilities to remove OTA in wines. Current clarification

products (organic or inorganic fining agents) have varying efficiencies for reducing OTA contents. Activated carbon and potassium caseinate were reported as interesting to remove OTA in wine. Potassium caseinate at high doses (150 g/hL) removed up to 82% of OTA, whereas activated carbon showed the highest specific adsorption capacity due to a high surface area per mass (Castellari et al. 2001) but could also modify phenolics composition in wine. Bentonite in white wines and yeast hulls in red wines have been described as the most effective noncarbonaceous fining agents for the removal of OTA (Leong et al. 2006a,b). However, sensorial repercussions on wine quality as well as allergenic reactions for some sensitive wine consumers cannot be totally excluded when using products to remove OTA.

Chitin and chitosan and some of their derivatives are nontoxic, biodegradable polymers that can remove organic contaminants (OTA) from food products (Bornet and Teissedre 2005). The results show a dose dependance with the higher removal of OTA occurring at 500 g/hL than at 200 g/hL. For 500 g/hL treatments, the levels of OTA in red wine was reduced by 83.4% with chitosan, and 56.7% with chitin–glucan. In the case of white wine, the levels of OTA are reduced until 53.4% with chitosan and 64.5% with chitin–glucan. In the case of the sweet wine, the levels of OTA are reduced by 26.1% with chitosan, and 43.5% with chitin–glucan. Chitosan was approved as a food additive in Asian countries (e.g., Japan) and Norway, and several studies have shown that this compound is not toxic (Hwang and Damodaran 1995).

36.3.4 Complexing Agent for Heavy Metals and Major Metals

During winemaking and storage, contaminants can alter the stabilization and safety of wines. Contaminants such as iron, lead, and cadmium have to be controlled.

Lead (Pb) adversely affects multiple enzyme systems within the body, as any ligand with sulfhydryl groups is vulnerable. For example, the detrimental effect of lead on the production of heme is well known (Idel'son 1968). Lead interferes with the critical phases of the dehydration of aminolevulinic acid and the incorporation of iron into the protoporphyrin molecule; the result is a decrease in heme production. Because heme is essential for cellular oxidation, deficiencies have far-reaching effects. Although lead can be excreted by the kidney, its elimination rate varies depending on the tissue that absorbed the lead. The reduction of lead levels in foods and beverages is therefore a necessity to improve food safety (Teissedre 1993). The OIV (Vine and Wine International Organization) established the maximum concentration of lead in wine at 150 µg/L. Wines contain small amount of rhamnogalacturonan-II dimer able to form strong complexes *in vitro* with lead and other selected cations (Pellerin et al. 1997). This pectic polysaccharide was shown to reduce intestinal absorption and tissue retention of lead in rats, therefore having a beneficial effect by reducing toxicity (Tahiri et al. 2000, 2002).

The toxic effects of cadmium (Cd) are due to the inactivation of enzymes containing sulfhydryl groups and the uncoupling of oxidative phosphorylation in mitochondria (Teissedre et al. 1994). Cadmium may also compete with other metals such as zinc and selenium for inclusion into metalloenzymes, and it may compete with calcium for binding sites on regulatory proteins such as calmodulin (Hu 1998). The OIV established the maximum level of cadmium in wine at 10 µg/L.

Unstable wines form different types of hazes. Excessive amounts of iron (>10–20 mg/L) oxidized to the ferric form can cause the precipitation of pigmented materials (blue haze) or orthophosphate ions (white haze). To produce a stable wine, the level of iron (Fe) must be inferior to 5 mg/L prior to bottling.

In a recent study, Bornet et al. (Bornet and Teissedre 2008) demonstrated that chitosan and chitin–glucan from fungal origin can reduce the levels of iron, and heavy metals (Pb, Cd), thereby improving wine safety and quality. Red, white, and sweet wines were spiked with either Fe (20 mg/L), or Pb (500 µg/L) and Cd (20 µg/L). The wines were then treated with chitosan and chitin–glucan, at a dose of 0.1, 0.5, and 2 g/L. Depending on the treatment, red wines showed reduction in iron concentration by 73%–90%, in cadmium concentration by 29%–57% and in

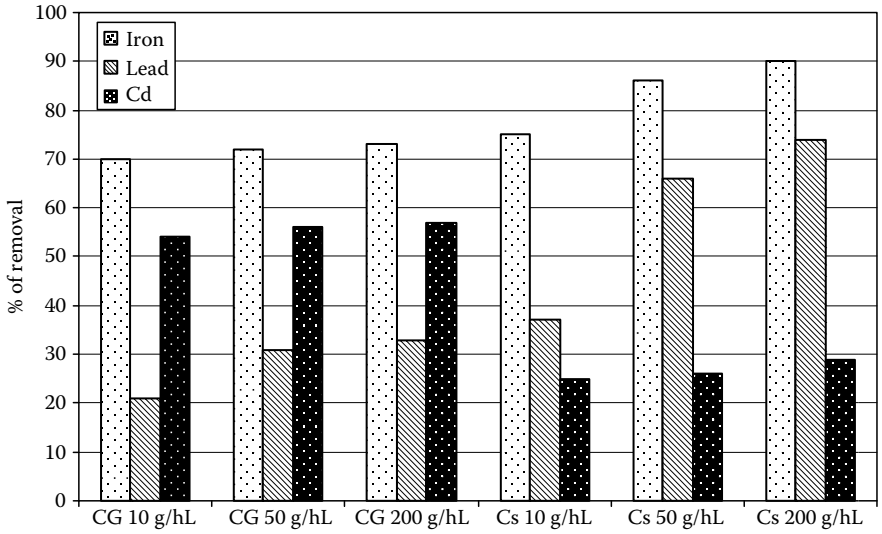

FIGURE 36.4 Effect of chitin–glucan (CG) and chitosan (Cs) on removal of iron and lead from wine (initial concentration of iron = 23 mg/L, initial concentration of cadmium = 19 μg/l, and initial concentration of lead = 150 μg/L, pH = 3.8).

lead concentration by 33%–74%. The same treatments on white wine decreased iron level by 32%–91%, cadmium level by 11%–23%, and lead level by 50%–65%. In the case of sweet wines, iron concentration was reduced by 51%–90%, cadmium concentration by 17%–25%, and lead concentration by 38%–84%. Results obtained with chitosan and chitin–glucan on the removal of iron and lead are presented in Figure 36.4.

36.4 APPLICATIONS IN OTHER BEVERAGE INDUSTRIES

36.4.1 Water Purification

The largest single use of chitosan is the clarification of waste and effluent water (Onsoyen et al. 1990). Better awareness of the ecological and health problems associated with heavy metals and pesticides and their accumulation through the food chain has prompted the demand for the purification of industrial water prior to their discharge for use. Conventional methods for the removal of metals from industrial wastewater may be ineffective or expensive, especially when metals are present at low concentrations. The ability of the amino groups of chitosan to form coordinate covalent bonds with metal ions is thus of great interest.

The use of commercially available chitosan for drinkable water purification has been approved by the United State Environmental Protection Agency (USEPA) up to a maximum level of 10 mg/L (Knorr 1986).

Chitosan, carboxymethyl chitosan, and cross-linked chitosan have been shown to be effective in the removal of Pb^{2+}, Cu^{2+}, and Cd^{2+} from drinking water (Muzzarelli et al. 1989; Tirmizi et al. 1996; Deans and Dixon 1992).

Chitosan is more efficient than activated charcoal for the purification of polychlorinated biphenyls from contaminated water (Thome et al. 1986).

36.4.2 Acidity-Adjusting Agent

Chitosan can be used for the deacidification of fruit juices Codex Alimentarius Commission (2003). Indeed chitosan salts carry a strong positive charge that can interact with proteins and hence act as

a dehazing agent in fruit juices (Shahidi et al. 1999). By the addition of chitosan into grapefruit juice at a concentration of 0.0015 g/mL, the total acid content was reduced by 52.6% due to a decrease in the amount of citric acid, tartaric acid, L-malic acid, oxalic acid, and ascorbic acid by 56.6%, 41.2%, 38.8%, 36.8 %, and 6.5%, respectively.

Scheruhn et al. (1999) reported that treating coffee drinks with chitosan increased the pH level and decreased the acid content of the beverage due to the acid-binding properties of chitosan with coffee. The efficacy of this treatment depends on the concentration, source, and processing of chitosan as well as on the acid content of the drinks (Scheruhn et al. 1999).

36.4.3 Antimicrobial Agent

The growing demand for foods without chemical preservatives has driven the research on new products with natural antimicrobial properties. In this context, the antifungal properties of chitosan are interesting for the food industry especially since chitosan is recognized as a safe biopolymer suitable for oral administration. Numerous studies have demonstrated that chitosan and its derivatives have various biological activities such as antimicrobial activity (Nishimura et al. 1985; Tokoro et al. 1989; Kobayashi et al. 1990; Saiki et al. 1990; Tsukada et al. 1990; Maeda et al. 1992; Shibata et al. 1997).

There are numerous papers describing the application of chitosan as a preservative in foods. For example, in apple juice, 15 yeasts and molds associated with food spoilage including *Mucor racemosus* and *Byssochlamys* spp. were inactivated by chitosan at various concentrations, pH values, and temperatures (Roller and Corvill 1999). In mayonnaise containing chitosan and 0.16% of acetic acid, 5 log CFU/g of *L. fructivorans* were inactivated, and the remaining bacteria were below the sensitivity limit of the plate counting technique within the duration of the experiment. *Z. bailii* counts were also reduced by approximately 1–2 log CFU/g within the first day of incubation at 25°C, but this was followed by growth on the subsequent days, giving an overall growth delay of 2 days (Roller and Corvill 2000). Dipping fresh UK pork sausages in 1% chitosan glutamate solution prior to storage at 7°C inhibited microbial growth and was shown to increase the shelf life by eight days (Sagoo et al. 2002). A Russian patent (RU2170022) describes the use of up to 0.1% chitosan combined with 0.1% sorbic acid and a third antibacterial agent as preservative and antioxidant in caviar.

36.5 CONCLUSIONS

Nowadays the food-processing industry is confronted with new statutory requirements to assure the safety of the consumers. In the beverage industry, the use of chitin and chitosan from fungal origin seems to be an alternative of choice as they are natural, biocompatible, and nonallergen.

Chitinous polymers are already used in

- Clarification of fruit juices: Chitosan at low concentrations was found to be effective.
- Clarification of white wines: The industrial tests showed that in most cases, an equivalent or even greater technological effect was obtained with chitin–glucan in comparison to common fining agents.
- Clarification of water and water effluents: Chitosan and chitin can be utilized as a tool for the purification of wastewater because of their high absorption capacity. The capacity of chitin and chitosan to form complexes with metal ions has been exploited in Japan for water purification.
- Prevention of oxidation and stabilization of the wine color: Chitosan has a good affinity to phenolic compounds, which are the main components involved in the wine oxidation processes and are responsible for browning. Chitosan reduced the polyphenol content and stabilized white wines to the same extent as did potassium caseinate, an adjuvant normally used in oenology. Moreover, chitosan could be reused after a simple regeneration process.

- Deacidification of drinks: Chitosan salts that carry a strong positive charge have been shown to be effective as dehazing agents. They may be used to control acidity in fruit juices or coffee drinks.
- Detoxification of the drinks (heavy metals, OTA): Chitosan and chitin–glucan are of real interest to reduce the levels of contaminants (iron, heavy metals, mycotoxins) especially in beverages. They should become auxiliary during production or storage to reduce the contribution of beverages to the dietary intake in contaminants. The toxicity of these polysaccharides is currently investigated in view to obtain their official use in the wine industry.
- Preservation of drinks from microbial deterioration such as *Brettanomyces*: The natural antimicrobial activity of chitin, chitosan, and their derivatives against different groups of microorganisms such as bacteria, yeast, and fungi has received considerable attention in recent years mostly because of the increasing concern of consumers regarding the safety and health benefits of the foods they eat.

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37 Chitin Nanofibrils and Their Derivatives as Cosmeceuticals

P. Morganti

CONTENTS

37.1 A YALURON-LIKE POLYGLUCOSIDE

Chitin is a sugar molecule, a copolymer of glucosamine and *N*-acetylglucosamine linked together by a typical β-1-4 bond. Extracted from by-products in the shell-crustaceous fish industry, it is the most abundant nitrogen-bearing organic component on earth (from 10^{10} to 10^{12} tons year), after cellulose (Muzzarelli 1993; Percot et al. 2003). Crustaceans, a class of marine organism, are characterized by the presence of an exoskeleton, necessary to protect them from predators. This multilayered composite tissue consists of a specific material providing mechanical support to the body, and enabling mobility through the formation of joints and attachment sides for muscles.

Organized to lies in protecting the inner organs from swelling in sea water, the exo- and endocuticle are composed of crystalline-alfa-nanofibrils chitin (CN) associated with various proteins and hardened by a considerable amount of calcium carbonate minerals. CN is thus an insoluble linear polymer easily available in nature, organized by polysaccharide chains arranged in different planes (Figure 37.1).

These chains twisted in a plywood-type pattern and with yaluron-like structure (Figure 37.2) have a reparative equilibrating role, due to their ability to entrap growth factors, active endogenous and exogenous substances, and a great quantity of water molecules (Muzzarelli et al. 1999; Raabe et al. 2006).

The extremely small size of these nano-crystals, separated from each other and bound with water, and their mostly electropositive relative electric charges, arranged along the axis of the molecule, allow this polyglucoside to remain stably suspended in water solutions, ready to bind with other molecules via ionic bonding (Revol et al. 1996) (Figure 37.3).

For these particular characteristics and for its bio- and eco-compatibility, CN may be used as a raw material in the pharmaceutical and dermocosmetic fields as well as to produce biofunctional textiles (Morganti et al. 2007a, Morganti 2008a).

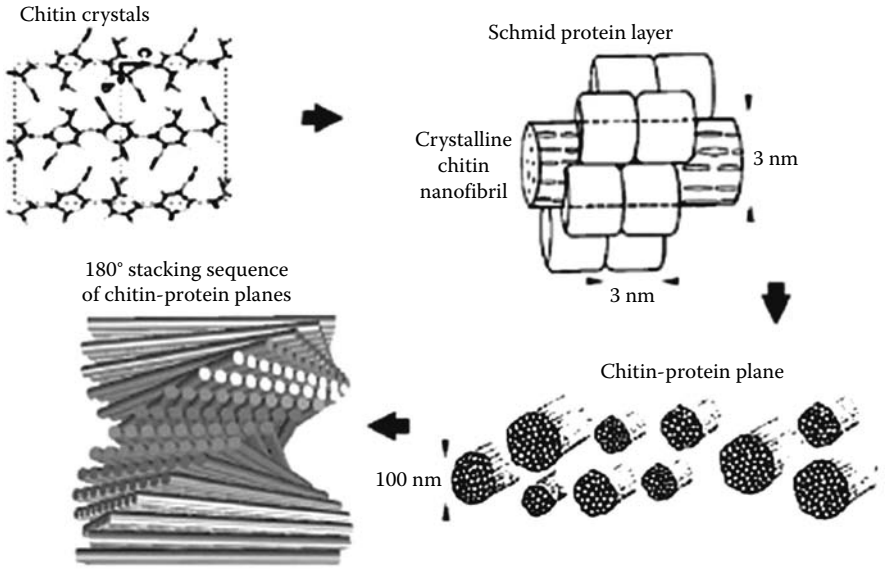

FIGURE 37.1 Crystalline nanofibrils of alfa-chitin organized by polysaccharides chains and arranged in different planes. (From Raabe, D. et al., *Mater. Sci. Eng. A*, 421, 143, 2006. With permission.)

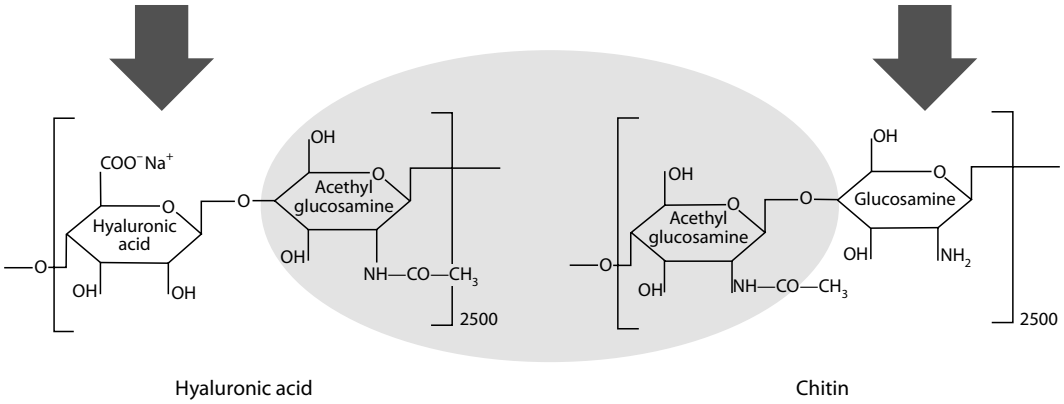

FIGURE 37.2 Hyaluronic acid and alfa-chitin have the same makeup.

37.2 CN: DIMENSION AND CHARACTERISTICS

These crystals are called *nano* due to their average size of 240 × 7 × 5 nanometers (nm), and *fibrils* because of their shape as thin needles. Moreover, they are *natural* because they are present in nature and *safe* to use because, being recognized by enzymes, they are *bio*- and eco-compatible.

These nanofibrils' average size being one-fourth of a bacterium, 1 g of product develops a surface of 400 mt^2 (Morganti et al. 2006a,b). Naturally, the types of compounds CN forms with antioxidants such as lutein, melatonin, lipoic acid, or with immune modulators as ectoin or beta-glucan, depends on the environment in which it reacts and consequently on the intermolecular ionic bonds it establishes with the various molecules. However, of fundamental importance is the carrier (micellary or lamellary) in which these compounds are inserted and which influences their thermodynamic activity and the consequent interaction with the skin (Morganti et al. 2006b). Being a pure polyglucoside, it does not cause allergic reactions, which on the contrary can by caused by the protein part of the fish shells that, however, is *totally eliminated* during the production process.

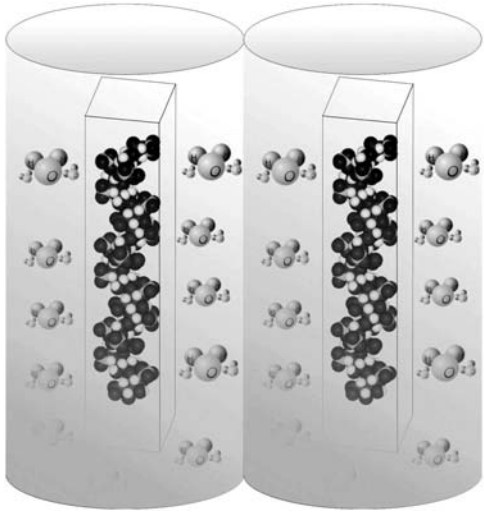

FIGURE 37.3 CN are separated from each other and bound with many molecules of water.

37.3 MECHANISM OF ACTION OF CN AND CN COMPLEXES

When linked with CN, the active principles show a more intense and effective biologic activity resulting in an increase in their bioavailability.

Naturally, the different activities the different CN compounds have is strictly dependent not only on the different ingredients used, but also on the different emulsions, micellar or lamellar, micro- and nano-structured realized. Depending on all these parameters, it is possible to obtain a transcutaneous, transdermal, or transfollicular penetration.

In this way, depending on the type of complex and the emulsions obtained, it is possible to produce innovative cosmetics able to achieve their specific activity at the level (Morganti 2007; Morganti and Morganti 2007a,b,c) of the stratum corneum (sunscreens products); the epidermis; and the dermis (antioxidant and antiaging cosmetics), specific cellular components like melanocytes (depigmenting agents), sweat glands (deodorants and antiperspirants), and hair follicles (anti–hair loss products).

However, it is possible to formulate multifunctional emulsions capable of being efficacious at different skin layers by the use of different active ingredients (Morganti et al. 2007b) (Figure 37.4).

In conclusion all these *new compounds* are able to function in a very specific way and with totally original mechanisms making them more effective and without collateral effects. Thus, due to their *high molecular weight* nanofibrils are unable to penetrate the inner cell, nor to enter the circulatory system, but they can be metabolized by skin enzymes and transformed into dimers or tetramers, with the subsequent formation of glucosamine and methyl glucosamine. These molecules can in turn be used exactly as they are or further catabolized to glucose and glutamic acid; the former takes part in cell energy production while the latter is a part of essential protein molecules (Sashiva et al., 1993; Kazel and Domenjoz, 1971).

37.4 BIOAVAILABILITY

The *bioavailability* of the different compounds of *CN-active substances*, fundamental for their effectiveness and safe usage, begins at the stratum corneum level, and goes to the cellular sites in response to the type of emulsions used (micellary or lamellary) (Morganti and Morganti 2008; Morganti et al. 2008a).

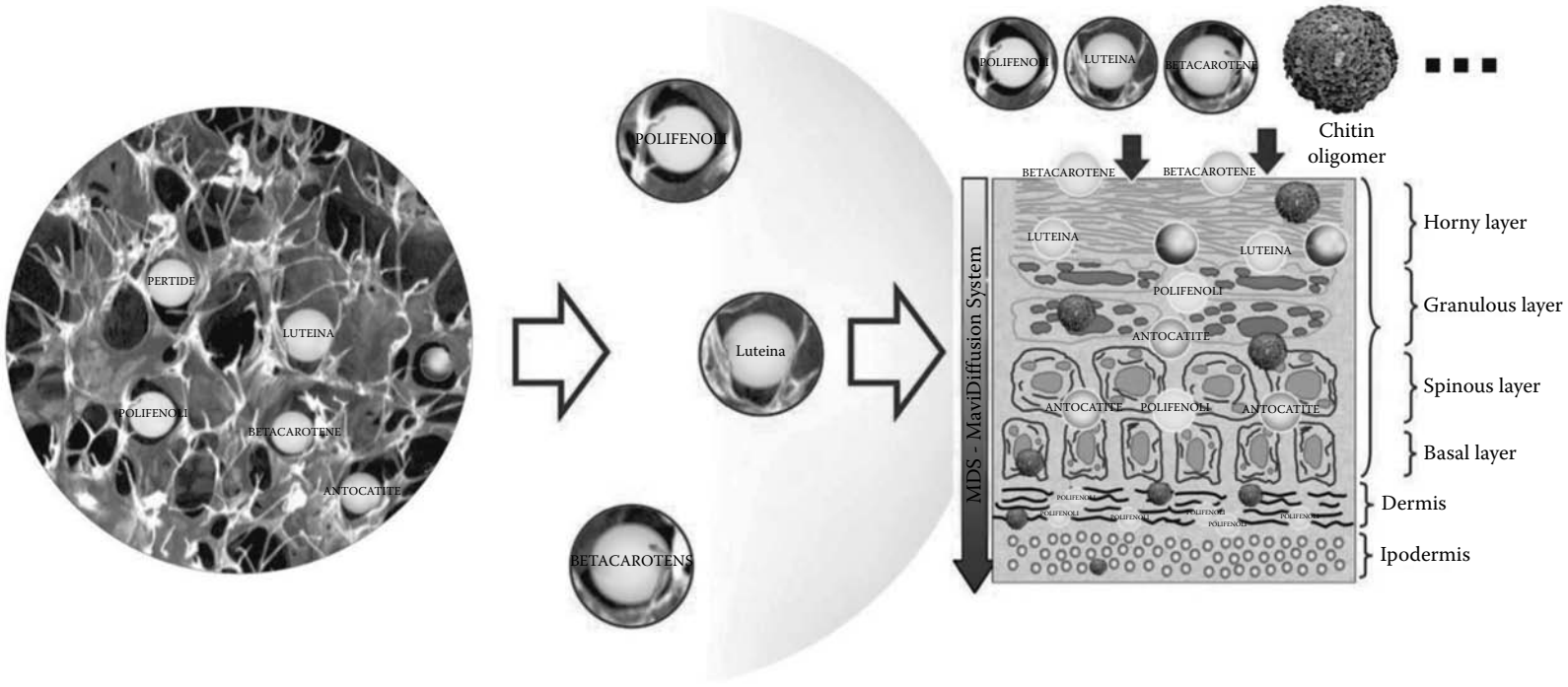

FIGURE 37.4 The various types of emulsions, whether micellary or lamellary, heavily influences an inter- or transcellular penetration.

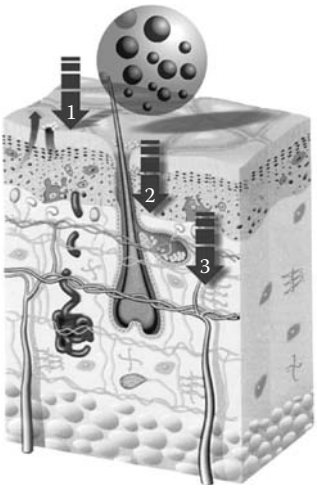

FIGURE 37.5 Transcutaneous penetration of chito-oligomers.

Stage I—On contact with the skin the polymer CN pre-bound with the active substance is transformed into *disaccharides* and/or *tetrasaccharides* (Figure 37.5).

Stage II—Disaccharides and tetrasaccharides, able to cross the stratum corneum will be localized in various regions of the epidermis and/or of the dermis ready to release the active substance combined with it and thereby perform its designated function.

The greater or lesser transcutaneous penetration will be directly tied to the size of the oligomer (whether dimer or tetramer) and the relative compound formed with the active substances, their thermodynamic activity, and, naturally, to the carrier used (Figure 37.6).

It is interesting to point out how, similar to what happens with hyaluronic acid, these dimers/tetramers seem able to enzymatically repolymerize themselves at the level of both the lipidic lamellae and the extracellular substance of the dermis. Later on, attracting and binding many water molecules, they act precisely like real natural sponges (Figure 37.7).

Besides disaccharide/tetrasaccharide repolymerization, a more complete CN hydrolysis can occur giving way to the formation of glucosamine and acetyl glucosamine that, as their precursors, can be used also for the synthesis of glycosaminoglycans, fundamental components of extracellular liquid. On the other hand, as described above, the glucosamine, synthesized by the body through the reaction between glucose and glutamic acid, can once more generate these two important molecules, glucose for energy and glutamic acid for protein building.

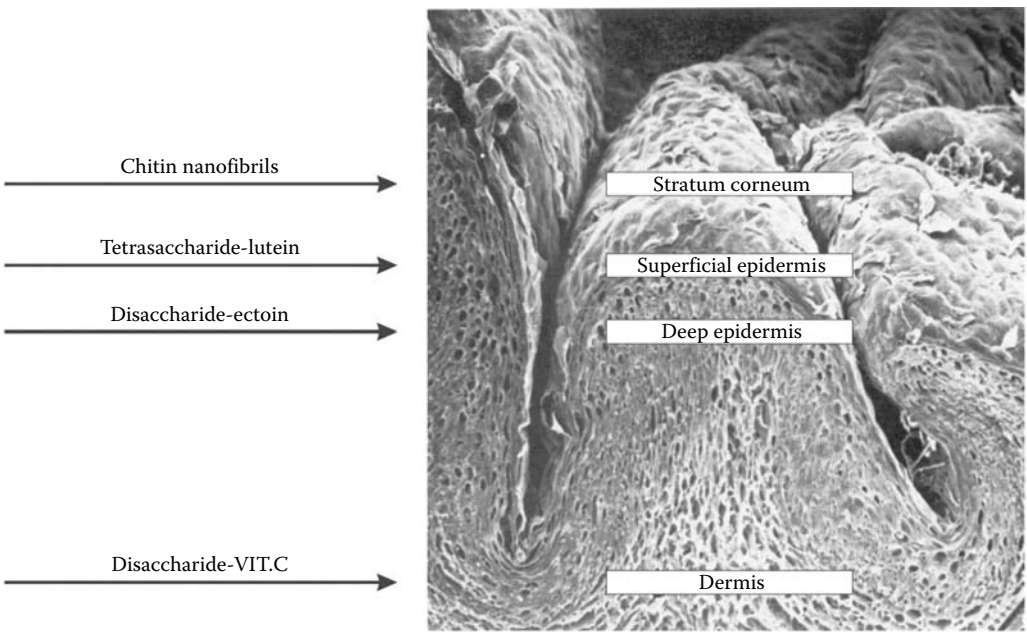

FIGURE 37.6 The different transepidermal or transdermal skin penetration and the relative product's effectiveness will be directly tied to the used emulsion, to the size of the chitin oligomer, and to the different bounds between the oligomer and the active compounds.

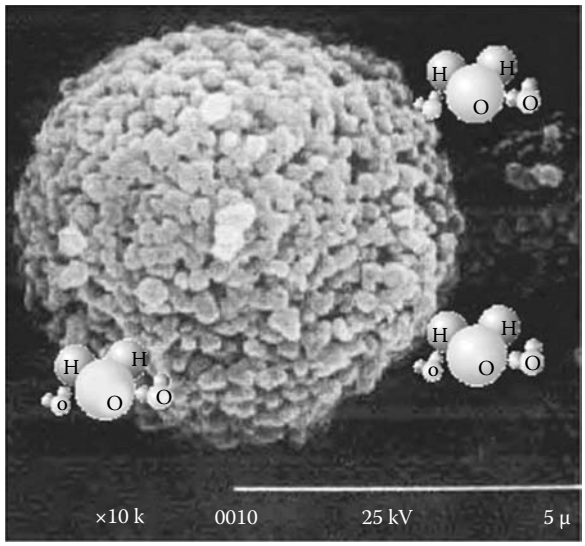

FIGURE 37.7 The CN hydrolyzed to dimers or to tetramers and binding with water increases the hydrating ability of the lipidic lamellae improving the skin barrier functions.

An interesting data seen in the degeneration of the cartilage of ligaments, tendons, and bone structures, besides that of elastic fibers, is that the enzyme elastase that plays a role in these pathologies increases its activity with aging.

Thus, the antiaging activity is recovered by the use of CN, first of all when they are used together with other active ingredients as melatonin and lutein (Biagini et al. 2000, 2007; Morganti et al. 2008b).

37.5 COSMETIC EFFECTIVENESS

By *in vitro* study (Morganti et al. 2008b,c) conducted with a lamellary cosmetic emulsion, it was possible to show the ability of CN to increase the reproduction of fibroblasts with a subsequent increase in collagen synthesis (Figure 37.8). This increase gave rise to an even greater production of ATP, necessary as the cell's energy source (Figure 37.9). As a consequence of the *in vitro* activity on photoaged subjects it was possible to obtain *in vivo:* an increase in skin hydration (Figure 37.10); an increase in surface lipids (Figure 37.11); the subsequent reduction of trans epidermal water loss (TEWL) (Figure 37.12); and a reduction of lipid peroxides (Figure 37.13).

These studies once more demonstrate the strict relationship between the activity carried out by the antioxidants, like melatonin and lutein especially if combined with CN, and the life of skin cells (Morganti et al. 2002; Morganti et al. 2004; Palombo et al. 2007; Jou et al. 2007; Reiter et al. 2008; Fischer et al. 2008; Berra and Rizzo, 2009). These molecules regulate, in fact, the rhythm of cellular growth or death, neutralizing free radicals and maintaining an efficient barrier. Thus, with the passing of time the mitochondria, the main electron transporters and enactors of the oxy/redox system, lose their efficiency in ATP synthesis bringing about an abnormal production of ROS (radical oxygen species) and of RNS (radical nitrogen species). For this reason, the cell, no longer able to provide for its own vital necessities, dies sooner (apoptosis). Furthermore, the deficit of ATP, the increase in free radical production, and the subsequent lack of water can induce a precocious apoptosis in mitochondria-dependent cells, as a result of the oxidative stress.

The results obtained showed an increased *in vitro* ATP production at the cellular level, accompanied by the *in vivo* improvement in hydration and skin barrier action, as well as the simultaneous reduction of lipid peroxides. These results showed how these particular types of cosmetic emulsions can be used to improve the condition of xerotic skin as well as skin damaged by photoaging (Morganti et al. 2008b,c).

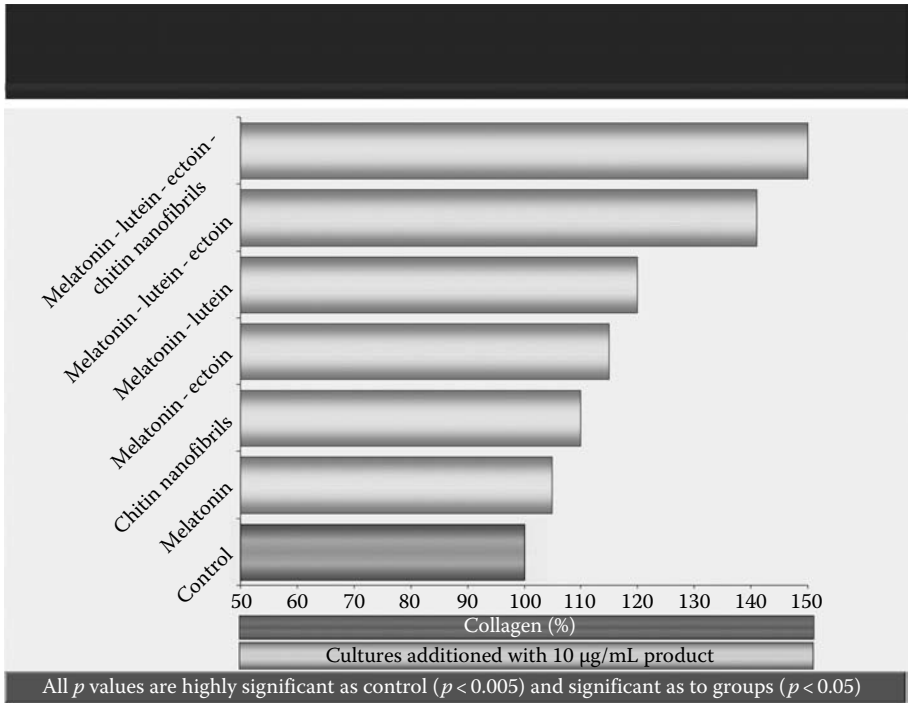

FIGURE 37.8 Increase in the collagen production of fibroblast cultures obtained by the use of CN in mixture with antioxidant and immunomodulant compounds.

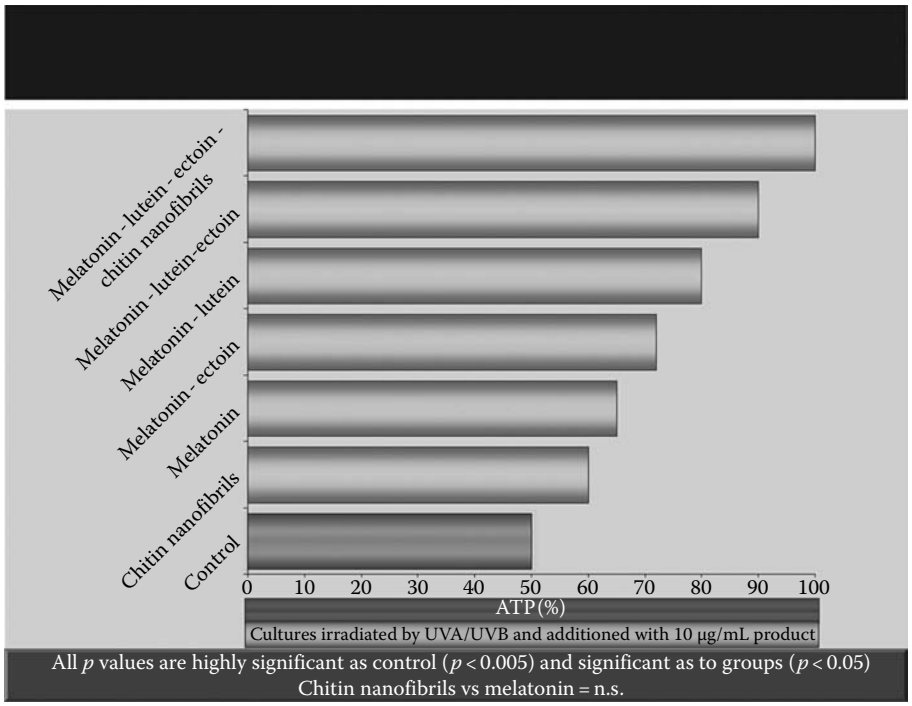

FIGURE 37.9 Protective activity on ATP production of irradiated keratinocyte cultures obtained by the use of CN in a mixture with antioxidant and immunomodulant compounds.

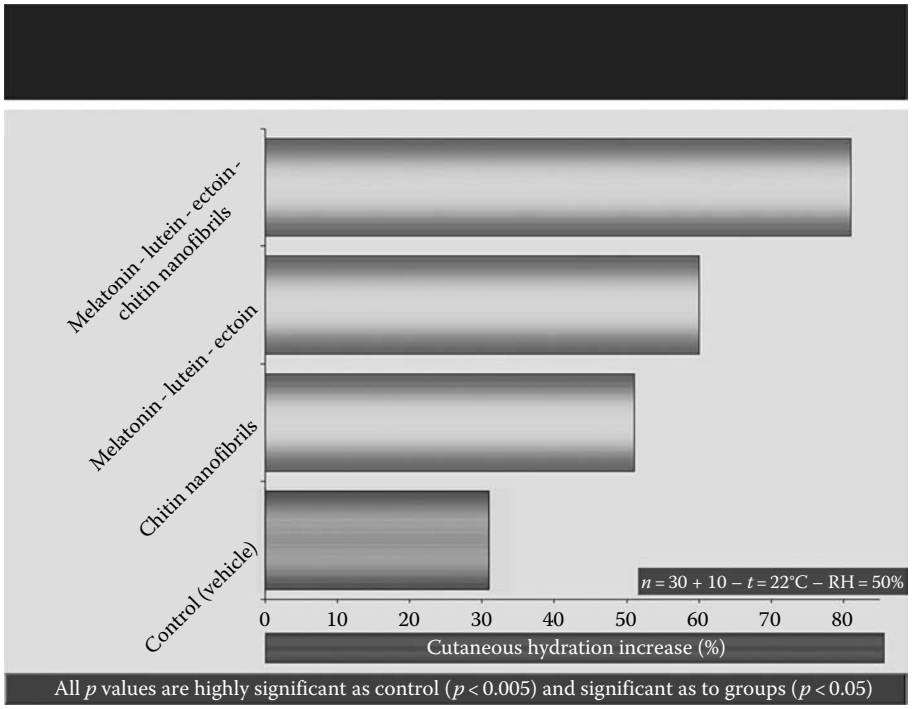

FIGURE 37.10 Activity of CN, antioxidant and immunomodulant compounds on skin hydration of women affected by dry skin after 60 days of bi-daily treatment on facial skin.

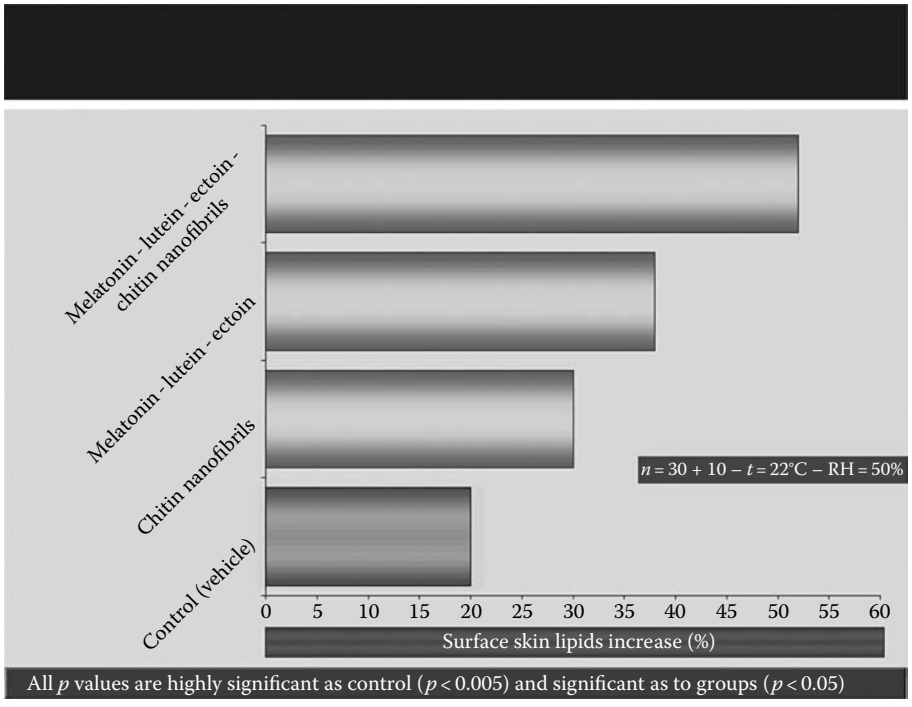

FIGURE 37.11 Activity of CN, antioxidant and immunomodulant compounds on skin surface lipids of women affected by dry skin after 60 days of bi-daily treatment on facial skin.

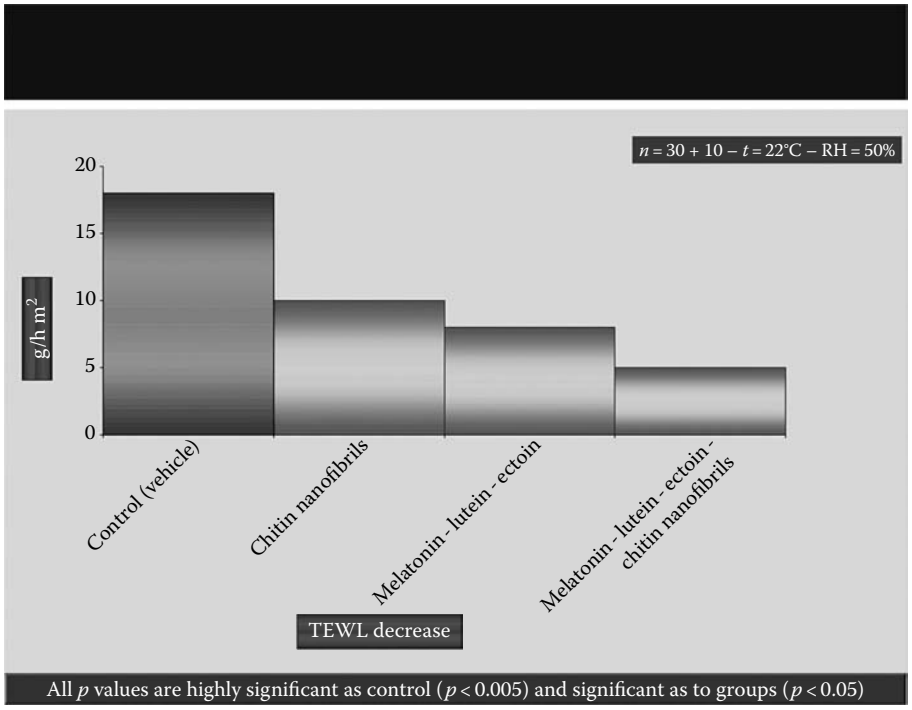

FIGURE 37.12 Activity of CN, antioxidant and immunomodulant compounds on TEWL recovered on women affected by dry skin after 60 days of bi-daily treatment on facial skin.

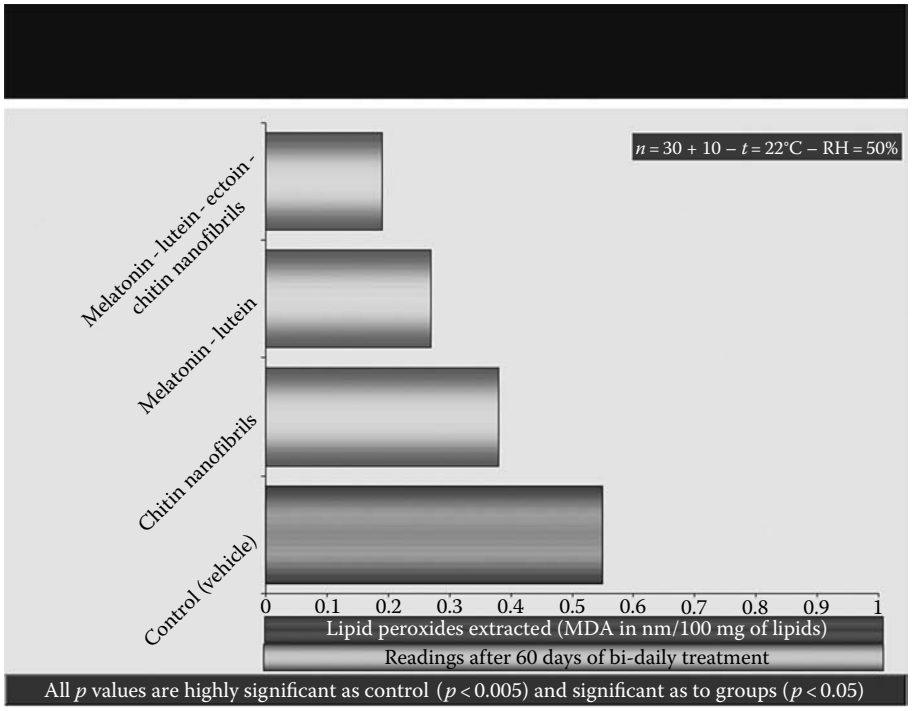

FIGURE 37.13 Activity of CN, antioxidant and immunomodulant compounds on lipid peroxides found on volar forearm of women affected by dry skin.

37.6 CONCLUSIONS

CN and their derivatives can be used in cosmetic dermatology to modulate the skin barrier activity and bettering the turnover of keratinocytes and fibroblasts. Moreover, their natural origin gives to CN a particular safeness and efficacy avoiding any kind of side effects.

Moreover, due to its facility to form complexes with a different range of molecules, CN seems to have the ability to bind triglycerides, fatty acids and bile acids, cholesterol, and other classes of lipids (Morganti et al. 2008b).

If these data are confirmed there will be the possibility to use CN as lipid-lowering compound in the obese subjects. These and other activities are subjects of our future studies.

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38 Chitin, Chitosan, and Their Oligosaccharides in Food Industry

Janak K. Vidanarachchi, Maheshika S. Kurukulasuriya, and Se-Kwon Kim

CONTENTS

38.1 INTRODUCTION

Chitin is the second most abundant polysaccharide found in nature after cellulose. The name "chitin" is derived from the Greek word "chiton," meaning a coat of a mail (Lower, 1984) or envelope (Tharanathan and Kittur, 2003). As the name implies, chitin is naturally found as the major structural component of the exoskeleton of invertebrates and in the cell walls of fungi. This linear

amino polysaccharide, which is named as poly β (1→4)-2-acetamido-2-deoxy-D-glucose, is composed of repeated units of 2-acetamido-2-deoxy-β-D-glucose linked by β (1→4) glycosidic bonds. The polysaccharide structure of the chitin is deacetylated into different degrees without destructing its polymeric chain for the production of chitosan. Hence, chitosan is composed primarily of glucosamine and 2-amino-2-deoxy-β-D-glucose and its polysaccharide structure is known as (1→4)-2-amino-2-deoxy-D-glucose. This complex structure has three types of reactive functional groups, an amino group at the C-2 position, and primary and secondary hydroxyl groups at C-3 and C-6 positions, respectively (Furusaki et al., 1996).

Both chitin and chitosan have unusual multifunctional properties, including high tensile strength, bioactivity, biodegradability (Simpson et al., 1994), biocompatibility, nonantigenicity, and nontoxicity (Tharanathan and Kittur, 2003), which made them possible to be used in many applications (Figure 38.1). Furthermore, the chemical modifications of the three reactive functional groups of chitosan have increased the applications of chitosan in different fields.

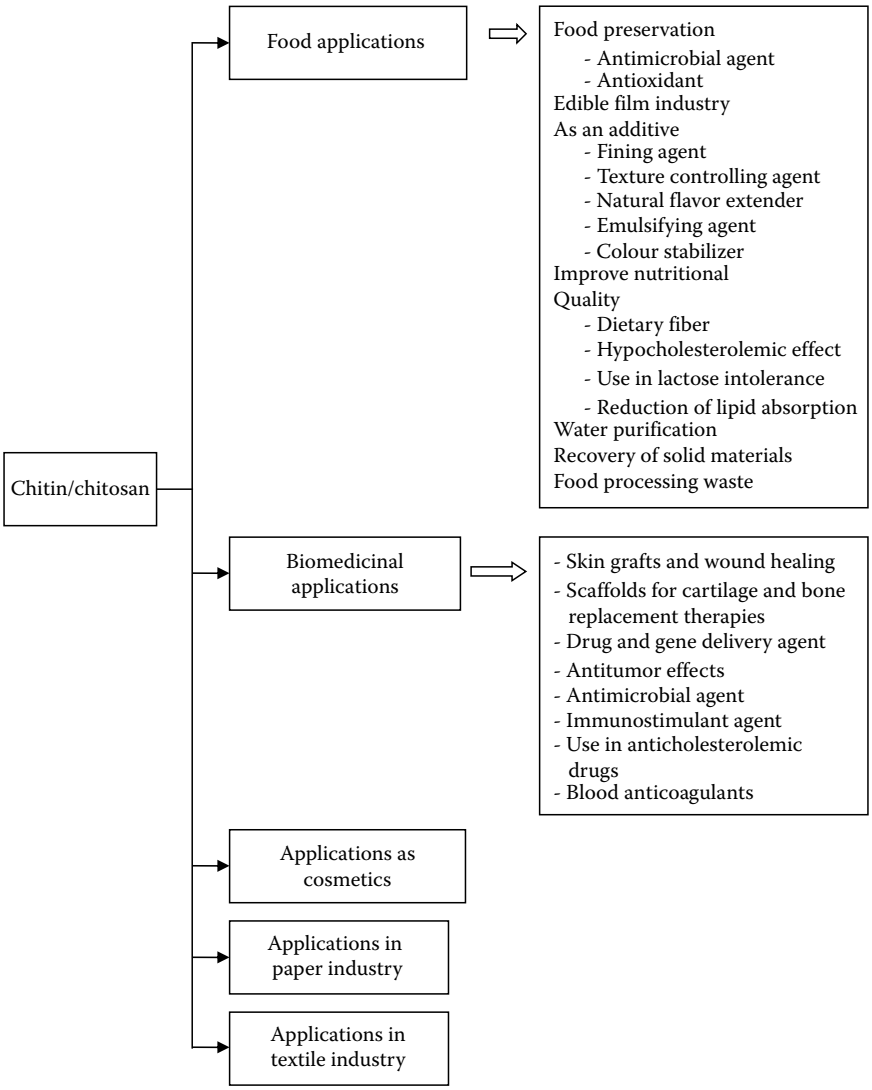

FIGURE 38.1 Applications of chitin and chitosan.

Even though chitin and chitosan have various properties that enable them to be applied in many fields, their poor solubility at neural pH due to high molecular weight and high viscosity limits their application in certain industries, especially in the food and biomedicinal industries.

However, chitosan oligosaccharides (COS), which are composed of 2–10 units of D-glucosamine units linked by β (1→4) glycosidic bonds, are readily soluble in water. This is due to their short chain lengths and the presence of free amino groups in the D-glucose units (Jeon et al., 2000). This greater solubility of COS at natural pH has attracted attention for the application of COS in different fields where poor solubility of chitin and chitosan has been a limiting factor, especially in the food industry. The special properties of COS have been applied for the improvement of food quality, food preservation, and for the use of COS as a functional food. Therefore, the objective of this chapter is to review the applications of chitin, chitosan, and their oligosaccharides in the food industry.

38.2 PREPARATION OF CHITOOLIGOSACCHARIDES

As chitin is found abundantly in the exoskeleton of crustaceans, the large quantity of crustacean shell waste from the seafood industry provides sufficient amount of raw materials for the synthesis of chitin and chitosan. The proteins, lipids, pigments, and calcium deposits in the crustacean shell waste are removed chemically, and chitin is extracted after passing through several steps. Then the chitin is *N*-acetylated by alkali treatment or acid treatment to form chitosan, which is more important for the production of COS (Figure 38.2). However, the preparation of

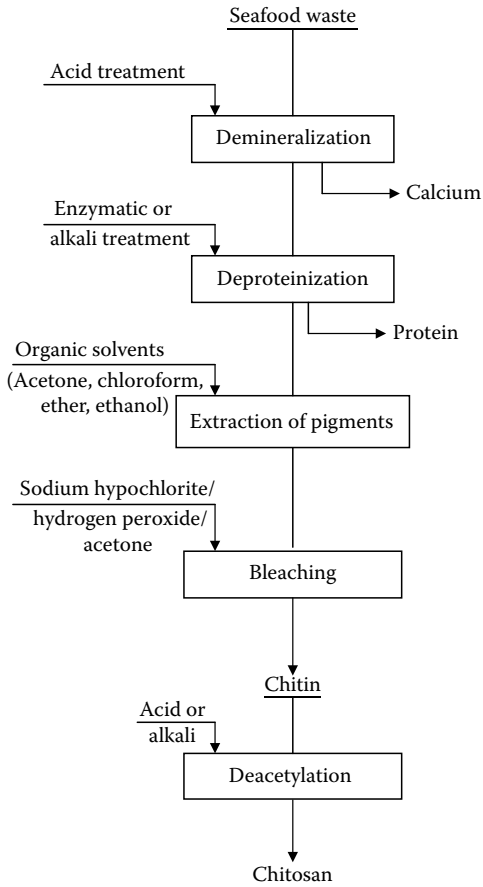

FIGURE 38.2 Flowchart of preparation of chitin and chitosan from seafood waste.

COS has been reported from both chitin (Rupley, 1964) and chitosan (Aiba, 1994; Jeon et al., 2000; Kuroiwa et al., 2003; Kou et al., 2004).

During the hydrolysis of chitin, only the glycosidic bonds between *N*-acetylated units have to be cleaved, whereas chitosan has four types of glycosidic bonds that have to be cleaved for the production of COS. These four types are the glycosidic bonds between two deacetylated units (D–D), deacetylated and acetylated units (D–A), acetylated and deacetylated units (A–D), and the bonds between two acetylated units (A–A). The energy required for the degradation of these bonds of chitin or chitosan is provided externally and the depolymerization of the polysaccharide chain into different degrees results in the COS with different molecular weights. These hydrolysis methods mainly include chemical hydrolysis by acids and the enzymatic hydrolysis.

38.2.1 Chemical Preparation of COS

The acid hydrolysis of chitin or chitosan is the conventional method of the chemical preparation of COS. In this method, acids are used for the depolymerization of the polysaccharide structure, resulting in the oligosaccharide form of chitin or chitosan. Rupley (1964) has depolymerized chitin with concentrated hydrochloric acid (Rupley, 1964) whereas Verum et al. (2001) have used both diluted and concentrated hydrochloric acid for acid hydrolysis. Recently, lactic acid has been used for the hydrolysis of chitosan (Il'ina and Varlamov, 2004). Despite the type or concentration of the acids, there is a high specificity in the acid hydrolysis reaction toward the type of glycosidic bond. The rate of cleavage of A–A and A–D linkages is high compared to the rate of cleavage of D–D or D–A linkages (Verum et al., 2001; Il'ina and Varlamov, 2004). Therefore, acid hydrolysis is more suitable for the production of chitin oligosaccharides and COS that are hydrolyzed from the initial material of less deacetylated chitosan. However, acid hydrolysis needs a large quantity of acid solutions, which is expensive and results in low yields of COS with large amounts of biologically inactive monomeric D-glucosamine units. The production of a high amount of monomeric D-glucosamine units is due to the difficulties in the control of the extent of acid hydrolysis, which should be ceased at the oligosaccharide stage. These drawbacks deviate the attention from acid hydrolysis toward the enzymatic hydrolysis, which is considered as the preferred method for the preparation of COS in large quantities.

38.2.2 Enzymatic Preparation of COS

Overcoming the problems of acid hydrolysis, scientists have adopted different enzymatic hydrolysis methods using different types of enzymes for the preparation of COS. The polysaccharide structure of the chitin or chitosan is converted to oligosaccharide form by specific enzymatic depolymerization or nonspecific enzymatic depolymerization.

38.2.2.1 Specific Enzymatic Hydrolysis

Chitinases and chitosanases are specific enzymes that are capable of cleaving the glycosidic bonds in the chitin and chitosan. Chitinases act specifically on the glycosidic bonds between the 2-acetamido-2-deoxy-β-D-glucose units of chitin and results in chitin oligosaccharides. Yamasaki et al. (1992) found the enzyme isolated from *Enterobacter* sp. G-1 only has the ability to hydrolyze chitin and chitosan that have less than 90% deacetylation. Furthermore, the hydroxylate of this experiment did not contain any water-soluble oligomers (Yamasaki et al., 1992), limiting the application of end results in the food industry. However, Mtisutomi et al. (1990) reported chitinases from *Aeromonas hydrophila* and *Streptomyces griseus*, which have affinity to the A–A and A–D linkages of the partially acetylated chitosan, respectively. Moreover, the resultant COS have shown important applications in the food industry due to the presence of free amino groups in their structure. As chitinases, chitosanases have also been purified from many microorganisms, including bacteria (Izume et al., 1992; Yamasaki et al., 1993). Unlike chitinases, chitosanases are specific toward the D–D bonds in

the chitosan molecules (Izume et al., 1992). The *Bacillus* sp. No. 7-M chitosanases act on the fully deacetylated chitosan resulting in COS from disaccharides to pentasaccharides with a high yield (Izume and Ohtaraka, 1987). Izume et al. (1992) used the same enzyme on the partially *N*-acetylated chitosan for the production of COS. According to the results, the mode of action of chitosanases on the partially *N*-acetylated chitosan is different from the mode of action of chitinases on it. The chitosanases are specific toward the D–D glycosidic bonds of chitosan whereas chitinases act on the A–D linkages. The increased production of oligosaccharides due to the increment in the degree of deacetylation (DD) of chitosan confirms the specificity of chitosanases toward the D–D glycosidic bonds.

38.2.2.2 Nonspecific Enzymatic Hydrolysis

Apart from the specific chitinases and chitosanases, nonspecific carbohydrases and proteases are capable of hydrolyzing chitosan. It has been found that the papain and pronase can be used for the chitosanolysis as a substitute for the expensive unavailable chitosanases as they result in the production of low molecular weight chitosan (LMWC), chitooligomers, and monomers (Kumar et al., 2005). However, the advantage of pronase as an enzyme for the catalysis of chitosan is the production of LMWC in high yields, which is difficult to achieve from the more specific chitosanases (Kumar et al., 2004). Therefore, the pronase would be more applicable for use in the column reactor part of the dual reactor system where LMWC is produced prior to the production of COS. Apart from the above nonspecific enzymes, researches have found that the pectinases isozyme of *Aspergillus niger* is also having a nonspecific chitosanolysis activity resulting in both LMWC and chitooligosaccharides, which can be applied in the food industry (Kittur et al., 2003).

All these enzymes have different optimum conditions needed to be maintained for the enzymatic reaction to occur. Different systems have been developed by scientists for the production of COS, efficiently providing the necessary conditions for the enzymatic reaction.

38.2.2.3 Batch Reactors

The batch reactors are the simplest method used for the enzymatic production of COS at the early stages (Izume and Ohtaraka, 1987). The enzyme is mixed with the substrate, chitin, or chitosan, and the optimum conditions are provided to the reaction mixture. According to Yamasaki et al. (1992), the chitosanases from *Enterobacter* sp. G-1 were stable at the pH range of 4–8 and the temperature up to 50°C. Similarly, Jeon and Kim (2000a) observed that chitooligosaccharides can be obtained by treating 1% chitosan with chitosanases from *Bacillus pumilus* BN-262 in a batch reactor. They observed that the optimum temperature and pH for the reaction were 45°C and 5.5, respectively. Although Yamasaki et al. (1992) have reported that the chitosanases are stable at high pH as 8, the experiments conducted by Izume and Ohtaraka (1987), Izume et al. (1992), and Jeon and Kim (2000a) have used the pH values 5.4, 6, and 5.5, respectively, which are more toward the acidic pH for the production of COS. The slight acidic pH may be more appropriate for the enzymatic reaction as the chitosan is soluble in slightly acidic solutions. At the initial stage of the reaction, Izume et al. (1992) as well as Jeon and Kim (2000a) have observed an increase in the rate of hydrolysis of chitosan. However, the same authors found that the rate of hydrolysis reached a maximum level and remained constant after several hours. Therefore, in the batch reactor method, the enzyme is inactivated by boiling for several minutes after the reaction reached its maximum level. This prevents the production of COS continuously as well as the reuse of enzyme, since the protein structure of the enzyme gets denatured by boiling. Therefore, the cost of production of chitooligosaccharides using batch reactor system is high. The yield of the final product is also low and it contains a mixture of COS with different molecular weights due to difficulties in the control of reaction in the batch reactor system. Therefore, attention has been paid on the development of an enzymatic reaction system that has the controlling ability of enzyme–substrate reaction.

Furthermore, the noxious odor of hydroxylate of batch reactor system due to the presence of inactivated enzyme limits its valuable food applications. As a solution, the immobilized enzymes have been introduced for the production of COS which could be removed from the mixture at appropriate

time. After removal, these immobilized enzymes have the advantage of reusability as they are not denatured after the enzymatic reaction. Different materials such as agar gel (Kuroiwa et al., 2003), chitopearl, calcium alginate gel (Yamasaki et al., 1992), and agar gel with Sepharose 6B (Kuroiwa, 2002) have been tested as materials for the immobilization of chitosanases. However, the enzyme immobilization material should be permeable to chitosan and the enzyme should adsorb to the material at the reaction pH to get the efficient production of COS.

38.2.2.4 Column Reactors

The continued preparation of COS is possible by packing the immobilized enzymes into a column reactor where substrate passed through. This system is similar to the packed-bed enzyme reactor (Kuroiwa et al., 2003) which has more advantages for obtaining desired COS due to their minimal axial mixing. Furthermore, Yamasaki et al. (1992) stated that the optimum conditions used in batch reactors with the immobilized chitosanases for the production of COS could also be used in the column reactors. According to their study, the immobilized enzymes were stable at the pH range of 4–6 and temperature up to 50°C which was the same temperature for the native-free enzymes. However, Kuroiwa et al. (2003) maintained the temperature at 35°C in packed-bed column reactor for the enzymatic reaction of chitosan and immobilized chitosanases during the production of COS. Usually the temperature in the column reactors is maintained by the circulation of water in the jacket surrounding the reactor at optimum temperature. When the enzymatic reaction reaches a steady state, the rate of input of the substrate and the rate of output of COS products are maintained at a same rate for its continued operation. Though this method has several advantages over the batch reactors, the poor affinity of immobilized enzymes to chitosan/substrate than that of free enzyme limits the activity of enzyme. These drawbacks limit the usage of column reactors in the commercial preparation of COS.

38.2.2.5 Ultrafiltration Membrane Reactor

The ultrafiltration membrane reactor system has been developed by overcoming the problems of the reusability of enzymes in batch reactors and the poor affinity of the substrate toward immobilized enzymes in column reactor systems. The substrate is supplied to the reaction vessel where free enzyme is added for the hydrolysis of chitosan. Then the mixture is sent through a column and allowed to get filtered through the ultrafiltration membrane. The chitooligosaccharides produced from the enzymatic reaction get separated from the substrate and the enzyme by getting filtered through the ultrafiltration membrane. The substrate and the enzyme remaining in the column are recycled to the reaction vessel for the next cycle of enzymatic reaction. Jeon and Kim (2000a) used an ultrafiltration membrane with the molecular weight cutoff (MWCO) of 3,000 Da, while Kou et al. (2004) filtered with MWCO of 10,000 Da for the separation of COS from the enzyme–substrate mixture. According to Jeon and Kim (2000a), the optimum conditions for the production of COS in the ultrafiltration membrane reactor system were with the recycling time of 180 min and permeation rate of 4 mL/min. Similar results were also observed by Kou et al. (2004), and the enzymatic reaction has reached the steady state within about 180 min from which the enzyme–substrate mixture can be separated from the produced COS for recycling. However, Kou et al. (2004) found that the enzyme/substrate ratio and residence time are the major factors that affect the performance of the ultrafiltration membrane reactor.

Compared to the systems described above, the membrane reactor system has the advantage of continued operation. However, the decrease in the enzymatic reaction and an increase in the transmembrane pressure was detected after five cycles (Jeon and Kim, 2000a; Kou et al., 2004). These researches have found that the membrane reactor could be operated continuously for at least 15 h, maintaining a constant permeate flux and product output rate (Kou et al., 2004). The continued production of ultrafiltration membrane reactor system gets obstructed due to membrane fouling after several cycles. Therefore, scientists' interest has moved toward the development of a new system that can be helpful in the efficient production of COS continuously.

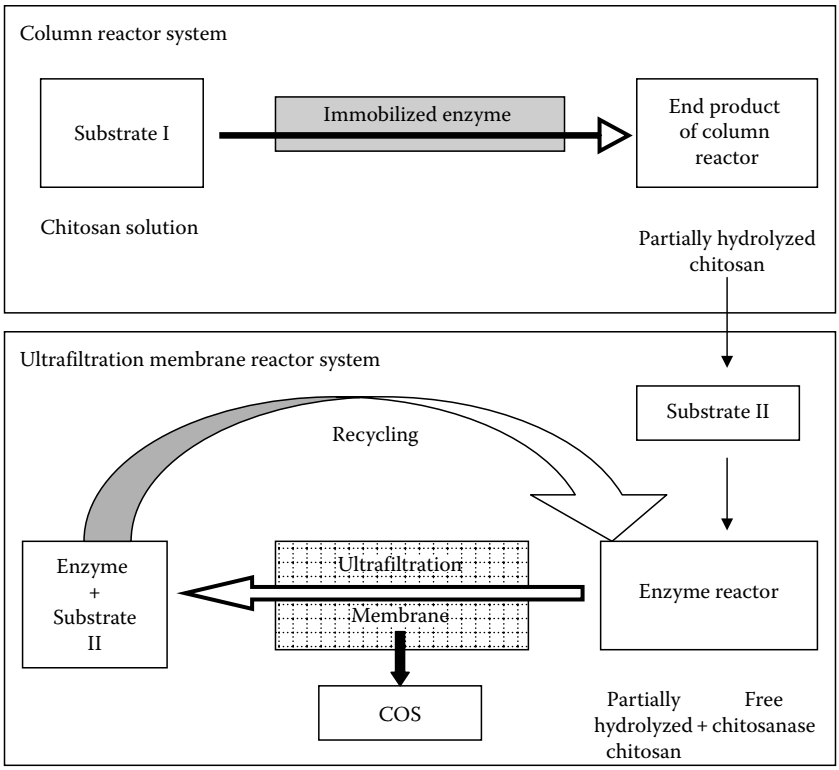

FIGURE 38.3 Mechanism of dual reactor system. (Adapted from Jeon, Y.J. and Kim, S.K., *Process Biochem*., 35, 623, 2000b.)

38.2.2.6 Dual Reactor System

The dual reactor system is composed of a column reactor packed with immobilized enzymes and an ultrafiltration membrane reactor (Figure 38.3), and this method has been adopted to solve the problem of membrane fouling in the membrane reactor system. Membrane fouling occurs due to the high viscosity of chitosan, which depends on the molecular weight. Reducing the molecular weight of chitosan by partial hydrolysis in the column reactor decreases the viscosity of substrate for the membrane reactor system, preventing membrane fouling. The column reactor of the dual reactor system hydrolyzes chitosan partially by immobilized chitosanases. The partially hydrolyzed chitosan, which has low viscosity, is presented to the membrane reactor system as its substrate. Therefore, the dual reactor system can be operated continuously for the production of COS without the problem of membrane fouling (Jeon and Kim, 2000b).

38.3 PHYSICOCHEMICAL PROPERTIES OF CHITOOLIGOSACCHARIDES

Usually a mixture of different chitooligosaccharides results from the hydrolysis of chitosan. The physicochemical properties of these COS increase their biological activities, enabling them to be used in various applications. The molecules with a molecular weight of 10 kDa or less in hydroxylate are considered as COS (Kim and Rajapakse, 2005). The molecular weight is an important factor that determines the solubility of chitosanolytic products. The COS is more soluble than the LMWC as they have a lower molecular weight than the LMWC. The solubility of COS increases with the degree of depolymerization (DP). Chitooligosaccharides that have depolymerized into smaller molecular size than the pentasaccharides or hexasaccharides are highly soluble in aqueous solutions and rapidly get absorbed while COS larger than the hexasaccharides are less soluble in the

aqueous solutions. Though solubility is high, the functional properties of those smaller molecular size COS are not high. The pentameric or hexameric COS have a balance between their physiological activity and the solubility. Therefore, these two forms of chitooligosaccharides are more important in the industrial applications. The uncontrollable hydrolysis of chitosan results in a high amount of physiologically less active chitooligosaccharides. Kuroiwa et al. (2003) have used immobilized chitosanases in a packed-bed enzyme reactor system for improvement in the yield of physiologically active COS. These authors have found that this system is able to produce pentamers and hexamers of COS for 1 month continuously.

Apart from the molecular size, molecular confirmation is also important for the biological activities of COS. Similar to chitosan, positive charges are present in the COS due to the deacetylation. Therefore, COS exhibit some of the properties present in chitosan. Chitooligosaccharides can strongly bind to the negatively charged surfaces due the presence of positive charges in its structure and display many biological activities.

These physicochemical properties of COS such as molecular weight, DD, and charge distribution enhance the antimicrobial activity, antioxidant activity, and nutritional quality of food, which are important in the food industry. Moreover, nontoxicity is the property of COS which ensures that the COS-added food products are safe for human consumption (Qin et al. 2008).

38.4 APPLICATIONS OF CHITOOLIGOSACCHARIDES IN FOOD INDUSTRY

Shahidi et al. (1999) have reviewed the food applications of chitin and chitosan, and these two biopolymers offer a wide range of applications in the food industry, including bioconversion for the production of value-added food products (Revah-Moiseev and Carroad, 1981; Shahidi and Synowiecki, 1991), food preservation (Ghaouth et al., 1992; Fang et al., 1994), formation of biodegradable films (Butler et al., 1996; Chen and Hwa, 1996; Kittur et al., 1998), recovery of waste material from food processing discards (Bough and Landes, 1976; Pinotti et al., 1997), water purification (Micera et al., 1986; Muzzarelli et al., 1989), and clarification and deacidification of fruit juices (Imeri and Knorr, 1988; Chen and Li, 1996; Rwan and Wu, 1996). However, COS have received attention due to their special properties which does not carry by the chitin or chitosan. These special properties of COS have been applied in food preservation, the use of COS as functional foods, and improvement in the quality of the animal origin foods.

38.4.1 Food Preservation

The microbial deterioration and oxidation of foods are the major problems that arise in increasing the shelf life of foods. However, food preservation has been achieved successfully with the chemical preservatives. The growing consumer demand toward foods without chemical preservatives has received attention due to natural preservatives, including chitin, chitosan, and COS. The special properties such as antimicrobial activity and antioxidative activity allow the chitin, chitosan, and especially COS to get successful as natural preservatives in the food industry.

38.4.2 Antimicrobial Activity of Chitooligosaccharides

Different microorganisms, including bacteria, fungi, and yeast, are responsible for the microbial deterioration of foods and they act as food pathogens as well. Though chitosan also exhibits antimicrobial property, the greater affinity of the COS against the microorganisms and greater solubility increase the possibilities of COS to be applied as a natural food preservative. The COS can act against both bacteria and fungi that are involved in the spoilage of food. However, scientists have found that the antibacterial activity of the COS is high compared to their antifungal activity (Wang et al., 2007a).

38.4.3 Antibacterial Activity of Chitooligosaccharides

Both Gram-positive and Gram-negative bacteria are involved in the spoilage of food. The type of bacteria varies with the type of food as the optimum conditions needed for the bacterial growth differ with their species. The chitosan and its oligosaccharides have the property of antibacterial activity against the wide range of food spoilage and food pathogenic bacteria which reduce the shelf life of food. These antibacterial activities of chitosan and COS depend mainly on their structure. The DP as well as the DD of the structure of chitosan and COS have an influence on their antibacterial activity.

The DP of these biomolecules is a major factor which changes their molecular weight or molecular size. The LMWC or COS with low DP exerts their antibacterial activity by getting easily penetrated through the cell membrane of microorganisms, disrupting the DNA replication leading to the suppression of the growth of microorganisms, whereas larger size molecules could not be penetrated through the cell membranes of microorganisms for the exertion of their antibacterial effects. Though there is a relationship between the molecular weight of chitosan and their antibacterial activity, Liu et al. (2006) have investigated that this relationship can get disturbed by the concentration of chitosan. Findings of Liu et al. (2006) indicated that there is no relationship between the antibacterial activity and molecular weight of chitosan at the higher (200, 5,00, 1,000 ppm) and lower (20 ppm) concentrations, whereas at the concentration from 50 to 100 ppm, the antibacterial effects of chitosan increase with the decrease in molecular weight.

In contrast to the antibacterial activity of chitosan, the antibacterial activity of COS decreased with the decrease in molecular weight (Rao et al., 2008), and among the chitooligomers, the maximum antibacterial effect was observed in hexamer followed by the penta, tetra, tri, and dimmers (Kittur et al., 2005). Recently, Wang et al. (2007a) observed that among COS having different DP, the highest antibacterial activity shown by the COS is having the DP of 4. The same authors also reported a reduction of antibacterial activity with the increase in DP of COS. These differences in the antibacterial effects of the above two experiments can be due to the other structural differences in the COS molecules, including the DD.

The deacetylation of chitin or chitosan results in free amine groups in the structure of chitosan or COS, making them effective against the bacteria. The involvement of the free amine group in the antibacterial activity of COS has been explained by the results of Kumar et al. (2005) as the *N*-acetylated chitosan oligomers have not shown any antibacterial activity while deacetylated chitosan oligomers, which have the free amine groups, showed an inhibition of bacteria depending on the concentration. These free amine groups present chitosan and COS act on the Gram-positive and Gram-negative bacteria by two different mechanisms. This difference is mainly due to the difference in the cell wall constituents of the two types of bacteria. The positively charged amino groups of chitosan or COS bind with the peptidoglycans in the cell walls of Gram-positive bacteria, resulting in cell wall disruption leading to the exposure of cell membrane to the osmotic shock. In Gram-negative bacteria, the positively charged amino groups of COS bind to the lipo-polysaccharides in the outer membrane by the ionic type of binding, blocking the nutrient flow leading to the death of Gram-negative bacteria. These two different action patterns of free amine group in the COS against the Gram-positive and Gram-negative bacteria have been explained by the results of the Kumar et al. (2005). In this experiment, proteins were detected in the chitooligomeric–monomeric mixture which has been treated with Gram-positive bacteria, *Bacillus cereus*, whereas no proteins were detected in the sample of Gram-negative bacteria, *E. coli*, reflecting the release of cell constituents to the media in the Gram-positive bacteria due to the disruption of their cell wall by interaction with free amino groups. However, despite the type of bacteria, the presence of free amine groups due to the deacetylation of chitosan or chitooligosaccharides favors their antibacterial activity, and with the increase of DD their antibacterial activity has also been increased. Furthermore, Wang et al. (2007a) reported that for an effective inhibition of bacteria DD of chitosan or COS should be higher than or around 80%.

The antibacterial activity of chitosan against the food spoilage and food pathogenic bacteria has been studied by many researches (Darmadj and Izumimoto, 1994; Zheng and Zhu, 2003). However, the antibacterial activities of COS compared to the chitosan are in debate, having both positive and negative points. No et al. (2002) have investigated that the antibacterial activity of chitosan is higher compared to the chitosan oligomers against the bacteria isolated from Tofu. This was in accordance with the results of Tsai et al. (2004) who have observed the strong antibacterial activity of LMWC at 100 ppm against many pathogens, including *Bacillus cereus*, *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Salmonella enteric*a *serovar Typhi*. In both cases, the antibacterial activity of chitooligosaccharides is much weaker compared to the chitosan. In the former experiment, chitosan oligomers showed antibacterial activity only against *Bacillus* sp., among the four species of bacteria that were tested. Similarly, in a recent study Rao et al. (2008) found that the antibacterial activity of the chitosan is higher compared to COS against *E. coli*, *Pseudomonas fluorescens*, *S. aureus,* and *B. cereus*. Furthermore, Zivanovic et al. (2004) also observed that the COS was less effective against all the tested bacteria than chitosan in oil-in-water emulsions.

Though the antibacterial effects of COS in the above experiments were lower than the chitosan, chitooligosaccharides have also shown the capability of inhibiting the growth of bacteria, including *Salmonella Typhi*, *S. aureus,* and lactic acid bacteria (Jeon et al., 2001). These researches further stated that the COS with molecular weight over 10,000 Da is required for the effective antibacterial activity.

In contrast to the above results, some researches have found an efficient bactericidal effect of chitooligosaccharide mixture compared to native chitosan (Kittur et al., 2005; Kumar et al., 2005; Wang et al., 2007a). Kittur et al. (2005) observed the efficient bactericidal effects of COS on Gram-positive and Gram-negative bacteria, *Bacillus cereus* and *E. coli*. Similarly, Kumar et al. (2005) observed the antibacterial activity of COS against *Yersinia enterocolitica* and *Bacillus licheniformis* other than the above-listed two species. Furthermore, Lin et al. (2009) have investigated the effect of chitooligosaccharides obtained by 24 h hydrolysis of chitosan with 90% DD on Gram-positive and Gram-negative bacteria, and according to this study chitooligosaccharides have been effective against both Gram-positive and Gram-negative bacteria with the Gram-negative bacteria having much lower minimum inhibitory concentrations (MIC) than the Gram-positive bacteria. Table 38.1 shows the MIC of COS needed for the different bacteria.

Though the study by Lin et al. (2009) has found a strong antibacterial activity of COS against the Gram-positive *Staphylococcus xylosus* and an effective inhibitory activity of COS against some of the Gram-positive bacteria (Table 38.1), the COS with 90% DD was not effective against *S. aureus* while COS with 83% DD was not effective against *Streptococcus bovis* and *S. pyogenes*, which belong to the category of Gram-positive bacteria. Similar to this study, Zheng and Zhu (2003) have found that chitosan having molecular weight less than 5 kDa is not effective against Gram-positive, *Staphylococcus aureus*. However, an effective antibacterial activity against *S. aureus* has been achieved by chitosan as it has shown a stronger antibacterial activity than COS (Fernandes et al., 2008).

Although chitosan has shown high antibacterial activity, No et al. (2002) found that the preparation of 1% chitosan solution was problematic due to its high viscosity. In order to overcome this problem, some researches have prepared water-soluble chitosan with 50% DD (Qin et al., 2006). However, the water-soluble chitosan and chitooligomers of this study had no significant antibacterial activity against *S. aureus* or *E. coli*, limiting its food applications. Therefore, chitooligosaccharides have attracted more attention to be applied as a natural food preservative. Furthermore, the level of antibacterial effect of water-insoluble chitosan can be achieved by increasing the concentration of water-soluble COS because the antibacterial effect depends on the concentration of COS in the medium (Jeon and Kim, 2000a; Zivanovic et al., 2004). Therefore, the high concentrations of COS with increased DD and optimum DP have the potential to be used in the food preservation as they are having an efficient antibacterial activity.

TABLE 38.1
Minimum Inhibitory Concentrations of Chitooligosaccharides for Different Bacteria

Type of Bacteria	Bacterial Strain	DD	MIC	Reference
Gram positive	*S. aureus*	NA	0.12 ± 0.01[a]	Wang et al. (2007a)
	S. aureus	90%	NI[b]	Lin et al. (2009)
	S. xylosus	90%	45 ± 10[b]	Lin et al. (2009)
	S. lactis	NA	0.12 ± 0.00[a]	Wang et al. (2007a)
	S. agalactiae	90%	800 ± 0[b]	Lin et al. (2009)
	S. bovis	90%	1440 ± 358[b]	Lin et al. (2009)
	S. pyogenes	90%	960 ± 358[b]	Lin et al. (2009)
	B. subtilis	NA	0.12 ± 0.01[a]	Wang et al. (2007a)
	L. monocytogenes	90%	880 ± 438[b]	Lin et al. (2009)
	L. monocytogenes	87.4%	2.5[c]	Benabbou et al. (2009)
Gram negative	*E. coli*	NA	0.08 ± 0.00[a]	Wang et al. (2007a)
	E. coli	90%	55 ± 27[b]	Lin et al. (2009)
	P. aeruginosa	90%	200 ± 122[b]	Lin et al. (2009)
	Y. enterocolitica	90%	880 ± 438[b]	Lin et al. (2009)
	V. harveyi	90%	60 ± 22[b]	Lin et al. (2009)
	V. parahaemolyticus	90%	55 ± 27[b]	Lin et al. (2009)

Note: NA, not available; NI, no inhibition.

[a] MIC as %.

[b] MIC as μg/mL.

[c] MIC as mg/mL.

38.4.4 Antifungal Activity of Chitooligosaccharides

As the bacteria, many fungi are involved in the postharvest putrefaction of the food items and acting as food pathogens. Numerous studies have been conducted for the investigation of the antifungal activities of chitosan and chitooligosaccharides and their potential to be applied as food preservatives. Though the scientists have found that the antimicrobial effect of chitosan or COS is higher against the bacteria than the fungi (Wang et al., 2007), the presence of antifungal properties may be important for the improvement of the shelf life of food items.

However, an antifungal study that had been conducted using yeast species showed the growth promotion activity of chitooligosaccharides on *Candida albicans* (Qin et al., 2006). This was in accordance with the results of Seyfarth et al. (2008) which have been observed as weak or no antifungal activities against the three yeast species, *C. albicans*, *C. krusei*, and *C. glabrata*. Though these experiments revealed the absence of the effect of COS against the fungi, a greater antifungal effect has been observed at higher concentrations of chitosan against the two postharvest fungal pathogens in strawberry, *Botrytis cinerea* and *Rhizopus stolonifer* (Ghaouth et al., 1992). Recently, Tsai et al. (2004) have also observed a strong activity of LMWC against the *Saccharomyces cerevisiae* while the chitooligosaccharides have much weaker antifungal activity.

The observations of the absence of antifungal effects of COS by Qin et al. (2006) and Seyfarth et al. (2008) may be due to the composition of COS mixture as Oliveira et al. (2008) have observed different levels of antifungal effects on the same species of fungi according to the composition of the COS mixture. The growth of *Botrytis cinerea*, *Rhizopus stolonifer*, and *Alternaria alternate* has been inhibited by two COS mixtures containing oligosaccharides of DP 2 to DP 10 and DP 2 to DP 12. The growth inhibition has not been reported against the *Botrytis cinerea* whereas the latter two species have showed a growth stimulatory effect with the COS mixture of DP 5 to DP 8.

TABLE 38.2
Minimum Inhibitory Concentrations of Chitooligosaccharides for Different Fungi

Fungal Strain	MIC (%)	Reference
Aspergillus niger	0.15 ± 0.01	Wang et al. (2007a)
Saccharomyces cerevisiae	0.13 ± 0.00	Wang et al. (2007a)
Rhizopus apiculatus	0.15 ± 0.00	Wang et al. (2007a)
Mucor circinelloides	0.15 ± 0.00	Wang et al. (2007a)

In the same study, weaker response has been reported from the *Penicillium expansum* by the COS mixture of oligosaccharides with DP 2 to DP 10 whereas other two mixtures had no effect (Oliveira et al., 2008).

Furthermore, the effects of DP on the antifungal activities of COS have been studied in detail by the scientists (Wang et al., 2007a). Among the different COS samples used in this experiment, the COS sample with DP 4 showed the maximum growth inhibition against the tested fungal species. The same authors have also observed decrease in the inhibition of fungal growth activity with increase in the DP of COS. The minimum concentration of COS mixture needed for the inhibition of the fungi varies with the species of fungi. Table 38.2 shows the MIC of chitooligosaccharides required by the different species of fungi which are acting as food pathogens and food spoilage organisms.

In contrast to the observations made in many studies, Wang et al. (2007a) reported that the antifungal effects of COS are higher than that of chitosan, and COS obtained from the hydrolysis of chitosan using the enzymes from *Pseudomonas* have the potential of use in aquatic food product preservation (Wang et al., 2007a).

38.4.5 Antioxidative Activity of Chitooligosaccharides

The oxidative changes that occur in the food items reduce the shelf life of foods, making them unsuitable for human consumption. Therefore, the antioxidative properties of COS also can be applied for the preservation of food.

The effect of the molecular weight of chitosan in the prevention of the lipid oxidation of salmon has been investigated (Kim and Thomas, 2007) and, according to this study, 30 kDa chitosan has exhibited the highest reduction of lipid oxidation in salmon. With the increase in the molecular weight of chitosan, the effectiveness against the lipid oxidation reduces reflecting the unsuitability of higher molecular weight chitosan as the food preservative.

Similar to chitosan, chitooligosaccharides produced by Gamma irradiation have exhibited the antioxidant property, increasing the potential of the usage of COS as a natural food preservative (Rao et al., 2006, 2008). According to the observations of Rao et al. (2008), among the different molecular weight chitooligosaccharides, COS with the molecular weight of 8.3 kDa exhibited the highest antioxidant potential in free radical scavenging assay. In the former experiment, the antioxidant lipid peroxidation activity of the COS has been investigated using the food models. In this experiment, lower thiobarbituric acid reactive substances (TBARS) have been detected in the meat samples coated with the COS than the COS uncoated samples after a storage period of 4 weeks in the ambient temperature, reflecting the involvement of COS in the improvement of the shelf life of meat. A significantly high antioxidant activity of COS has also been reported by Wang et al. (2007a) in the assay of antilipid peroxidation.

The structure of the chitooligosaccharides favors its antioxidant activities. The presence of functionally active two hydroxyl groups at C-3 and C-6 positions and a free amino group at C-2 position contribute to their antioxidant activity as those groups have abstractable hydrogen atoms

(Je et al., 2004; Park et al., 2004). The structural factors affecting the radical scavenging activity of chitooligosaccharides have been investigated by Huang et al. (2006). According to this study, COS can exert different mechanisms to scavenge the different free radicals. Furthermore, same authors suggested that the hydrogen donation is dominant for the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and carbon-centered radicals. Moreover, the chelation of ferrous ions has been indirectly contributed for the hydroxyl radical scavenging activity of COS. However, Rao et al. (2006) have stated that the ability of the antioxidant activity of COS is mainly related to their superoxide radical scavenging activity while hydroxyl radical scavenging activity is showed only up to 50%. Similarly, high superoxide radical scavenging activity has been reported by Wang et al. (2007) among the three marine oligosaccharides that have been tested.

Though the antimicrobial and the antioxidant properties of the chitosan and the COS are discussed separately, the combined effect of these two properties with the property of solubility is essential for the use of COS in food preservation. Moreover, scientists have investigated that synergistic effect can be obtained by combining the COS with other materials for the preservation of food. In the experiment of Rao et al. (2008), COS or lysozyme alone has no significant antibacterial effect against *E. coli* or *P. fluorescens* whereas a combination of COS and lysozyme enhanced the antibacterial activity against these two species. The inclusion of this mixture to minced meat eliminated the *E. coli*, *P. fluorescens*, and *B. cereus* from meat during 4 h at ambient temperature and no viable counts of those bacteria have been observed in the meat samples stored at chilled temperature even after 15 days (Rao et al., 2008).

38.5 APPLICATION OF CHITOOLIGOSACCHARIDES AS FUNCTIONAL FOODS

The consumer demand on the functional foods increases as they promote the gut health and decrease the risk of diseases. The functional foods are categorized into three groups based on their mechanism of action as probiotics, prebiotics, and biogenics (Mitsuoka, 2002). The chitooligosaccharides can also be applied as functional foods in the food industry as they have the capacity to act as a prebiotic.

38.5.1 Application of Chitooligosaccharides as Prebiotics

Prebiotics are nondigestible feed ingredient that beneficially affect the host by selectively stimulating the growth of beneficial bacteria and/or by suppressing the growth of harmful bacteria in the colon which have the potential to improve the host health (Gibson and Roberfroid, 1995). Usually, prebiotics are oligosaccharides. Therefore, COS also have the potential to be used as the prebiotics, increasing the application of COS as functional foods.

A study has been carried out for the investigation of growth stimulatory effects of COS on bifidobacteria and lactic acid bacteria using the fully deacetylated COS with the DP 2–8 (Lee et al., 2002). Though COS have exhibited bactericidal effects on certain lactic acid bacterial species in a previous study (Jeon et al., 2001), the bactericidal effects of COS against the bifidobacteria and lactic acid bacteria have not been reported in this study. The COS have a bifidogenic effect at concentrations between 0.1% and 0.5% whereas COS have a growth stimulatory effect on *L. casei* and *L. brevis* at a concentration of 0.1%. Since these chitooligosaccharides are fully deacetylated, they are not digestible by the intestinal enzymes increasing the applicability of COS as prebiotics.

Recently, Pan et al. (2009) have investigated the ability of COS to act as prebiotics in the mouse model system. The chiooligosaccharides used in this experiment were 90% deacetylated and the DP was ranged from 3 to 6. According to this study, the concentrations of more favorable bifidobacteria and lactobacilli increased while reducing the concentrations of unfavorable enterococcus and Enterobacteriaceae in the caecum of mice treated with COS for 14 days. According to these results, it can be suggested that the COS have the potential to be used as prebiotics in the food industry.

38.5.2 Hypocholesterolemic Foods

Hypocholesterolemic effects of the chitosan and COS have attracted much attention as this property can be applied widely in the biomedicinal field. However, according to an early study, chitosan oligomers have not shown a cholesterol-lowering activity (Sugano et al., 1988). Kim et al. (1998) have carried out an experiment for the investigation of the effects of hydrophilic chitooligosaccharides on the liver functions of mice. They have found that the COS can partially reduce the elevated serum cholesterol levels after a diet containing cholesterol. After the 6 weeks experimental period, the serum cholesterol level has been reduced by 23% in the group of mice fed with a diet containing 1% COS and 3% cholesterol than the group fed with 3% cholesterol alone (Kim et al., 1998). This reflects the involvement of the orally administered COS in the partial reduction of elevated serum cholesterol levels.

Choi et al. (2006) have used microencapsulated COS in milk, and according to this study the COS microcapsules can be used in commercial healthy milk production with little adverse effects on the physical and sensory properties. However, further studies are needed for the improvement of the physical and sensory properties of the hypocholesterolemic milk and the investigation of the possibility of production of other types of hypocholesterolemic foods for health benefits.

38.5.3 Enhancement of the Calcium Absorption

In the animal studies, scientists have found that the absorption of calcium is arrested by the dietary chitosan, limiting the applications of chitosan in the food industry (Liao et al., 2007). Though dietary calcium absorption is prevented by chitosan, its oligosaccharide form has been involved in the acceleration of the calcium absorption from the gut. This special property of COS can be applied in the food industry for the production of functional foods. Scientists have reported the property of the increased calcium absorption of COS in in vivo experiments (Jeon and Kim 1997). The chitosan oligomers from trimer to heptamer have been involved in increasing the calcium absorption, which is reflected by the lowering of the fecal calcium excretion and by an increase in the breaking force of femur in rats. Similar results have been reported by Jung et al. (2006). Furthermore, they have observed an efficient inhibition of the formation of insoluble calcium salts by chitooligosaccharides in the neutral pH in vitro experiments. Therefore, low molecular weight COS can be used as a dietary ingredient, as they are important for the prevention of negative mineral imbalances.

38.6 OTHER APPLICATIONS OF CHITOOLIGOSACCHARIDES IN FOOD INDUSTRY

Chitooligosaccharides can be applied in the improvement of the quality of the animal origin food items by introducing them to the animals as feed additives. The enhancement of the growth of the animal species, improvement of the animal gut health, and reduction of animal diseases may contribute to increase the application of COS in animal industries.

38.6.1 Effects of Chitooligosaccharides in the Improvement of Performance and Meat Quality

The effects of COS on the improvement of the performance of broilers (Zhou et al., 2009) as well as its applications in swine (Han et al., 2007; Liu et al., 2008) have been studied by the scientists.

Though there is no change in the feed conversion efficiency, body weight gain and feed intake have increased with the addition of COS into the diet of broilers. Zhou et al. (2009) have found that with increase in the concentration of COS in the diet, the weight of the abdominal organs such as liver increases while decreasing the abdominal fat and yellowness of the meat. Furthermore, the inclusion of COS in the diet of the broilers has improved the performance of the birds as well as the quality of the broiler meat.

In contrast to broilers, pigs have shown increased feed conversion efficiency due to the addition of COS in the diet. The digestibility of the feed has improved while decreasing the growth of harmful bacteria (Han et al., 2007). Similar results have been observed by Liu et al. (2008). Their results indicated that the dietary supplementation of 100–200 mg/kg COS enhanced the growth performance of pigs by increasing the digestibility, decreasing the incidence of diarrhea by enhancing the growth of favorable *Lactobacillus* species, and improving the small intestinal morphology. Therefore, the usage of COS as an animal feed additive improves the quality and quantity of animal origin foods.

38.6.2 Toxin-Binding Ability

The commercial toxin binder FERMKIT®, which contains chitin, chitosan, and COS fermented by probiotic bacteria, has been used as a dietary supplementation for ducks. Khajarem et al. (2003) have conducted an experiment for the investigation of the effects of the dietary supplementation of this product on the mycotoxicity in meat-type ducks. During the 4 weeks experimental period, the dietary supplementation of this product has significantly diminished the adverse effects of aflatoxin and zearalenone, which cause toxicity in ducks. Therefore, the usage of this product containing probiotic fermented chitin, chitosan, and COS as a dietary supplementation may improve the meat quality of ducks by preventing the possible mycotoxicities.

38.7 CONCLUSION

The second most abundant biopolymer chitin and its deacetylated form chitosan have a wide range of applications, including in the food industry. Insolubility at neutral pH is a major factor which limits the food applications of these two biopolymers and has attracted the attention of scientists toward the depolymerized oligosaccharide form of chitosan, which is soluble at neutral pH. Therefore, COS can be applied in the many fields where the insolubility of chitin or chitosan is becoming a limiting factor, especially in the food industry.

The low molecular weight due to less DP is the main factor which increases the solubility of COS. Though decrease in DP increases the solubility, the functional properties of COS are not high in small molecular size chitooligosaccharides. The pentameric or hexameric form of COS which has the balance between the solubility and physiological activities is more suitable for their food applications.

The food applications of COS include the preservation of food, the use of COS as functional foods, and the improvement of the quality of the animal origin foods. The chitooligosaccharides are used as natural food preservatives due to the presence of antimicrobial and antioxidative properties. However, their antimicrobial effects compared to the chitosan are in debate, having both positive and negative points. Moreover, the application of COS as functional foods due to the presence of prebiotic activity and hypocholesterolemic effects is a novel area which has a great potential to be developed. Further studies are needed for the investigation of molecular weight, molecular confirmation, and the mechanism of action of COS that contribute to their special properties, thereby widening the range of food applications of chitooligosaccharides.

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39 Chitin/Chitosan and Derivatives for Wastewater Treatment

P. N. Sudha

CONTENTS

39.1 INTRODUCTION

Water is a scarce resource. Only 0.03% of the world's water reserves are available for human activities. Population growth and industrial boom have increased the demand for water, while supply has remained constant. Moreover, inappropriate water management has reduced the amount of available resources (Allegre et al. 2004). As environmental protection is becoming an important global problem, industries are finding novel ways of developing technologies that do not cause environmental problems. There are several techniques used for wastewater treatment. The discharge of

wastewaters has become more and more stringent (Prigione et al. 2008). A number of processes have been tested for the decolorization of textile waters, including (a) sorption on activated carbon (Kavitha and Namasivayam 2008) or biosorbents (O'Mahony et al. 2002), (b) oxidation and photooxidation (Muruganandham and Swaminathan 2007), (c) chemical treatment (Gao et al. 2007), and (d) coagulation–flocculation (Koprivanac et al. 2000, Somasundaran and Runkana 2005, Zeng et al. 2007). The coagulation–flocculation process can be used as a pretreatment, a posttreatment, or even a main treatment (Gahr et al. 1994). But they are all expensive technologies. In recent times, polymers have been used for water management. The majority of commercial polymers and ion-exchange resins are derived from petroleum-based raw materials using processing chemistry that is not always safe or environment friendly.

Today, there is a growing interest in developing natural low-cost alternatives to synthetic polymers (Crini 2006). Chitin is a nontoxic, biodegradable polymer of high molecular weight. Chitosan is derived from a natural product, chitin by deacetylation. *The properties of chitin and chitosan enable them to attach to a variety of organic contaminants (bacteria, algae, urea, sweat), minerals, metals, and oil. Chitosan, therefore, dramatically increases the effectiveness of filtration systems, being sand or cartridges, which normally cannot capture fine particles and solved pollutant* (france-chitine.com). The annual worldwide chitin production has been estimated to be 10^{11} t, and industrial use has been estimated to be 10,000 t. If these waste products are used for wastewater treatment, it not only increases the potential of applications but also reduces the environmental pollution caused by the disposal of this underutilized by-products.

39.2 PREPARATION AND PROPERTIES OF CHITIN AND CHITOSAN

39.2.1 Chitin

Chitin is the second most ubiquitous natural polysaccharide after cellulose on earth, and is composed of β (1→4)–lined 2-acetamido–2-deoxy-β-D-glucose (Dutta et al. 2002) (*N*-acetylglucosamine) (Figure 39.1), which is white, hard, inelastic, nitrogenous polysaccharide with biodegradability, biocompatibility, nontoxicity, and metal adsorption (Hudson and Smith 1998). Chitin is easily obtained from crab or shrimp shells and fungal *mycelia*. In the first case, chitin production is associated with food industries such as shrimp canning (Madhavan 1992). Depending on its source, chitin occurs as two allomorphs, namely, the α and β forms (Blackwell 1973, Rudall and Kenchington 1973), which can be differentiated by infrared and solid-state NMR spectroscopy together with x-ray diffraction. A third allomorph γ-chitin has also been described (Rudall 1969, Rudall and Kenchington 1973). The weight-average molecular weight of chitin is 1.03×10^6–2.5×10^6, but the *N*-deacetylation reaction reduces this to 1×10^5 to 5×10^5 by HPLC and light-scattering techniques (Muzzarelli et al. 1987).

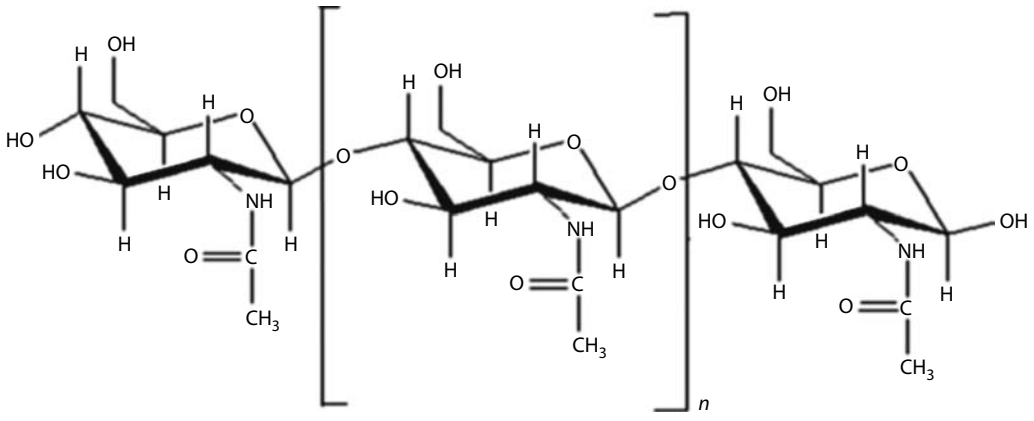

FIGURE 39.1 Structure of chitin.

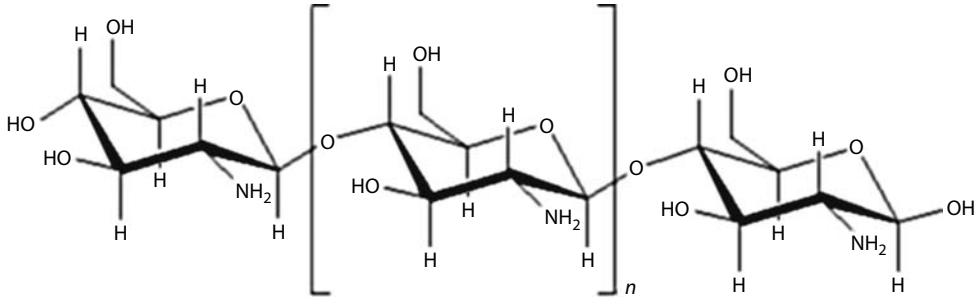

FIGURE 39.2 Structure of chito.

39.2.2 Chitosan

Chitosan, a partially *N*-deacetylated product of chitin carrying positive charges, is an important natural biopolymer (Figure 39.2) due to its biocompatibility and biodegradability (Henriksen et al. 1993, Peter 1995, Ohkawa et al. 2000) and broad applications in wastewater treatment (Mireles et al. 1992, Peter 1995, Torres et al. 1998, Savant and Torres 2000, Wibowo et al. 2005a,b), chemical industries (Chen 1999), biomedical and pharmaceutical applications (Lian and Chen 1998, Chen 1999, Lee et al. 2001, Wang et al. 2001), agriculture, and biotechnology (Peter 1995). Chitin, the starting compound of chitosan, can be degraded by the enzyme chitinase. Four main steps are involved in chronological order to produce chitosan from crustacean shells: (1) deproteinization, (2) demineralization, (3) decoloration, and (4) deacetylation. To produce 1 kg of 70% deacetylated chitosan from shrimp shells, 6.3 kg of HCl and 1.8 kg of NaOH are required in addition to nitrogen, process water (0.5 t), and cooling water (0.9 t) (Madhavan and Nair 1974). Chitosan is a hydrophilic, natural cationic polymer, and an effective ion exchanger, with a large number of amino groups, which are responsible for the high adsorption property of chitosan. The oxygen atom in the hydroxyl group is having less affinity for heavy metals according to the HSAB (hard and soft acids and bases) classification system (Winter 1994). If a soft ligand group such as sulfur is introduced on to the chitosan backbone, it will increase the uptake capacity for many heavy metals because cadmium can be classified as soft acids, which have a strong affinity to soft ligands. Since sulfur has a very strong affinity for most heavy metals, the metal–sulfur complex is very stable in basic conditions. The use of materials with sulfur functional groups, such as xanthate group, shows improved selectivity for the removal of heavy metals in wastewater (Sankararamkrishnan et al. 2005).

39.3 MODIFICATION OF CHITIN AND CHITOSAN

39.3.1 Modified Chitin and Chitosan

The poor solubility, low surface area, and porosity of chitin and chitosan are the major limiting factors in their utilization. Chitosan can be modified by physical or chemical processes in order to improve the mechanical and chemical properties. Chemical modification of chitosan has two main aims: (a) to improve the metal adsorption properties and (b) to change the solubility properties of chitosan in water or acidic medium. The substitution chemical reactions involve the NH_2 group in the C_2 position or the OH groups in the C_3 and C_6 positions of acetylated and deacetylated units. Chitosan membrane is swollen in water; the amino groups may be protonated and leave the hydroxide ions free in water, which may contribute to the ionic conduction in the membrane.

The chitosan derivatives mentioned in the literature (Muzzarelli 1985, Roberts 1992, Morimoto et al. 2002, Hudson and Jenkins 2003) show that one can differentiate specific reactions involving the $–NH_2$ group at the C-2 position or nonspecific reactions of –OH groups at the C-3 and C-6 positions (especially esterification and etherification) (Rinaudo and Reguant 2000). The $–NH_2$ in the

C-2 position is the important point of difference between chitosan and cellulose, where three –OH groups of nearly equal reactivity are available.

39.3.2 Physical Modification of Chitin and Chitosan

The efficiency of adsorption depends on physiochemical properties, mainly surface area, porosity, and particle size of adsorbents. Chitosan has a very low specific area ranging between 2 and $30\,m^2\,g^{-1}$ whereas most commercial-activated carbons range between 800 and $1500\,m^2\,g^{-1}$ (Crini 2005). Chitosan-based materials are used in different fields of application in the form of powder, flakes, and foremost as gels, beads, membrane, sponge, fibers, hollow fibers, etc. (Kas 1997, Merrifield et al. 2004, Guibal 2005, Krajewska 2005). Flake and powder forms of chitosan are not suitable to be used as adsorbents due to their low surface area and no porosity (Varma et al. 2004). Chitosan flakes modified into beads are essential for the enhancement of adsorption performance (Guibal 2004). Chitosan bead is an open porous system; as such there is every possibility that the dye may be transported within the bead through its porous network. However, cross section of Congo red–adsorbed beads showed no sign of intraparticle diffusion. Molecular shape and size are the prime parameters in the intraparticle diffusion process. In aqueous solutions, the amine groups of chitosan are much easier to cationize and they adsorb dye anions strongly by electrostatic attraction. However, pure chitosan has some disadvantages such as unsatisfactory mechanical properties, severe shrinkage, deformation after drying, solubility under acidic conditions, and compressibility at high operating pressure. Several methods to overcome these disadvantages have been performed by coating or impregnating chitosan on rigid, porous materials such as silica gel (Liu et al. 2004).

39.3.3 Chemical Modification of Chitin and Chitosan

Chitosan is a multi-nucleophilic polymer due to the presence of the NH_2 and OH functional groups. The initial sites where substitution occurs are the more nucleophilic amino groups. However, the experimental conditions and protection of the NH_2 groups reduces the intermolecular hydrogen bonding and creates space for water molecules to fill in and solvate the hydrophilic groups of the polymer backbone (Sashiwa and Shigemasa 1999). *N*-alkylated derivatives can be obtained by the treatment of chitosan with aldehydes or ketones via formation of Schiff base intermediates, aldimines (from reactions with aldehydes), or ketimines (from reactions with ketones) followed by reduction of the imine with sodium borohydride.

The positive charges on chitosan can also participate in ionic interactions, particularly with polyanions such as alginate, carrageen, and pectin. The complexes formed by electrostatic interaction between COO^- or SO_4^{2-} and NH_4^+ (Illum 1998) have been proposed for the recovery of suspended solids from aqueous food processing streams (Mireles et al. 1992, Mireles-De Witt 1994, Torres et al. 1998, Savant and Torres 2000, Savant 2001) that can be used for animal feed (Wibowo et al. 2005a,b).

Grafting of chitosan allows the formation of functional derivatives by covalent binding of a molecule, the graft, onto the chitosan backbone (Jayakumar et al. 2005). The properties of the resulting graft copolymers are controlled by the characteristics of the side chains, including molecular structure, length, and number (Mourya and Namdar 2008). The cross-linking agents can be of varying length and contain other functional groups than those involved in cross-linking (Merrifield 2002).

Partial cross-linking by di/polyfunctional reagents enables the use of chitosan for metal adsorption in acidic medium. Several bi- or polyfunctional cross-linking agents such as glutaraldehyde (GLA) (Monterio and Airoldi 1999, Jeon and Holl 2003, Osifo et al. 2008, Wan Ngah and Fatinathan 2008), ethylene glycol diglycidyl ether (EGDE) (Wan Nagh et al. 2002, Wan Nagh et al. 2005), glyoxal (Martinez et al. 2007), epichlorohydrin (EPI) (Baba et al. 1998, Vieira and Beppu 2005), benzoquinone (McAfee et al. 2001), and cyclodextrin (CD) (Sreenivasan 1998, Tojima et al. 1998, Martlet et al. 2001) have been used. The fact that the cross-linking agents cited before are neither

safe nor environment friendly has led to the use of water-soluble cross-linking agents such as sodium trimetaphosphate, sodium tripolyphosphate, or carboxylic acids (Crini 2005).

The amine group of chitosan is modified using many chemical methods including *O*- and *N*-carboxymethyl chitosans, chitosan 6-*O*-sulfate (Naggi et al. 1986, Terbojevich et al. 1989), and more recently as *N*-sulfated chitosan (Holme and Perlin 1997), *N*-methylene phosphonic chitosans (Heras et al. 2000, Ramos et al. 2003). Trimethylchitosan ammonium cationic derivative, water soluble at over all the practical pH range, is obtained by quaternization of chitosan (Domard et al. 1986). Chitosan-grafted copolymers poly (ethylene glycol)-grafted chitosan, (Morimoto et al. 2002), PEG can also be introduced by reductive amination of chitosan using PEG-aldehyde (Harris et al. 1984),

Alkylated chitosan (Desbrieres et al. 1996). This technique was also used to introduce *n*-lauryl chains (Muzzarelli et al. 2000). Alkylated chitosans with good solubility in acidic conditions (pH < 6) have a number of very interesting properties. Simple surfactant and modified chitosan have completely different behaviors (Babak et al. 1999, Nonaka et al. 2002). Chemical modification of the chitosan flakes with xanthate group increased the adsorption capacity to more than four times compared to the plain flakes. Adsorption followed Langmuir isotherm model. Adsorbent-adsorption kinetics exhibited pseudo second-order. Due to the high stability constant of cadmium with xanthate group, interference from common cations and other electrolytes like sulfate, chloride, and carbonate were not found (Sankararamakrishnan et al. 2007). So the functional group of chitosan, amine, and alcohol are easily modified by many organic reactions: tosylation (Kurita et al. 1991), alkylation (Kurita et al. 1990), carboxylation (Muzzaralli et al. 1982), sulfonation (Terbojevich et al. 1989), Schiff base (Moore and Roberts 1982), and quaternary salt (Muzzaralli and Tanfani 1985). The biosorbents possess an outstanding capacity and high rate of adsorption, and also high selectivity in detoxifying both very diluted or concentrated solutions. They also have a very high affinity to many varieties of dyes.

Carbohydrates can be grafted on the chitosan backbone at the C-2 positions by reductive alkylation: For that purpose, disaccharides (cellobiose, lactose, etc.) having a reducing end group, are introduced, in the presence of a reductant, on chitosan in the open-chain form (Yalpani and Hall 1984). These derivatives are water soluble. Fibers made of chitin and chitosan are useful as absorbable structures and wound-dressing materials (Nakajima et al. 1984, Muzzarelli 1997, Ravi Kumar 1999).

39.4 DIFFERENT FORMS OF CHITIN AND CHITOSAN

As chitosan is versatile, it can be manufactured into films, membranes, fibers, sponges, gels, beads, and nanoparticles, or supported on inert materials. The utilization of these materials presents many advantages in terms of applicability to a wide variety of process configurations. The various forms of chitin and chitosan are discussed below.

39.4.1 Fibers

The preparation of chitin threads for use in the fabrication of absorbable suture materials, dressings, and biodegradable substrates for the growth of human skin cells fibers has been reported (Tamura et al. 2004, Mikhailov and Lebedeva 2007). One study reports that chitin fibers have comparable properties to those of collagen and lactide fibers (Desai 2005). Melt spinning is ruled out as chitin decomposes prior to melting. Porous chitosan (CS) fibers have been shown to be useful as reinforcement in CS-based nerve conduits fabricated from CS yarns and a CS solution by combining an industrial braiding method with a mold casting/lyophilization technique (Wang et al. 2007).

39.4.2 Nanofiber

Nanofiber is defined as fiber with a diameter on the order of 100 nm. They can be produced by interfacial polymerization and electrospinning. The electrospinning of chitin was performed with

1,1,1,2,2,2-hexafluoro-2-propanol (HFIP) as a spinning solvent. Although as-spun chitin nanofibers have broad fiber diameter distribution, most of the fiber diameters are less than 100 nm. The chitosan nanofiber mats showed good erosion stability in water and high adsorption affinity for metal ions in an aqueous solution. The adsorption of Cu(II) and Pb(II) were fitted well with Langmuir isotherm indicating that monolayer adsorption occurred on the nanofiber mats (Sajjad Haider et al. 2009). The optimum conditions for the formation of chitosan nanofibers were investigated by Homayoni et al. (2009) and found that the diameter of the optimum product is 140 nm, which corresponds to chitosan hydrolyzed for 48 h with the molecular weight of 2.94×10^5 g mol^{-1}. These nanofibers present 13.95% moisture uptake, which is 74% higher than that of the polymer powders.

39.4.3 Hollow Fibers

The excellent mass-transfer properties conferred by the hollow fiber configuration soon led to numerous commercial applications in various fields such as the medical field (blood fractionation), water reclamation (purification and desalination), gas separation, azeotropic mixture separation (using pervaporation), and wastewater treatment. Conventional wastewater treatment processes are energy intensive and can emit, directly and indirectly, significant amounts of greenhouse gases. In order to increase the sustainability of wastewater treatment, new approaches are needed. An interesting approach is integrating hollow-fiber membranes (HFMs) into an activated sludge tank.

Chitosan, also in the form of HFM, can be used for wastewater treatment. Chitosan/cellulose acetate blend hollow fibers with a high chitosan content as adsorptive membrane can be prepared (Liu and Bai 2005) with a nonacidic organic dope solvent, *N*-methyl-2-pyrrolidone. blend HFMs have fast adsorption kinetics (reaching adsorption equilibrium in less than 3 h) and high adsorption capacity (up to 103–105 mg copper ions per gram of chitosan or 31 mg copper ions per gram of the blend HFMs with solution pH 5) attributed to the high chitosan content and possibly the improved accessibility of chitosan in the blend HFMs (Han et al. 2007). The separation performances of an aqueous ethanol solution through chitosan/PSf composite HFMs with the vapor permeation operating process were investigated. Compared with PSf HFMs, the chitosan/PSf composite HFMs are effectively able to increase the permselectivity of water (Tsai et al. 2006).

39.4.4 Membranes

Chitosan is a hydrophilic material and likely to impart the hydrophilicity to the membranes prepared by formation of its composite with another mechanically stronger and hydrophobic material such as poly (acrylonitrile) (PAN). In acidic pH range, chitosan will be positively charged due to protonation of $-NH_2$ groups and hence will exhibit electrostatic interactions with charged solutes such as proteins. Application of chitosan for ultrafiltration (UF) membranes has been reported by Aiba et al. (2007) and Kaminski and Modrzejewska (1997). There is no reported literature on PAN/chitosan composite UF membranes, although studies on PAN/chitosan composite pervaporation membranes are reported (Watanabe and Kyo 1992, Wang et al. 1996). Similarly, chitin membranes were prepared by Nagahama et al. (2008). These novel biodegradable chitin membranes were found to be promising biomaterials in the tissue engineering field.

39.5 CHITIN AND CHITOSAN FOR WASTEWATER TREATMENT

The environmental disposal of untreated effluents to its surroundings often leads to the following consequences: coloration of water bodies with the creation of an aesthetic problem limitation of reoxygenation capacity of the receiving water, decrease in sunlight penetration which in turn disturbs photosynthetic activities in the aquatic system, and chronic and acute toxicities (Kadirvelu et al. 2005, Arami et al. 2006). This diverse spectrum of wastes requiring efficient treatment has focused the attention of researchers on membrane, ion-exchange, and biological technologies.

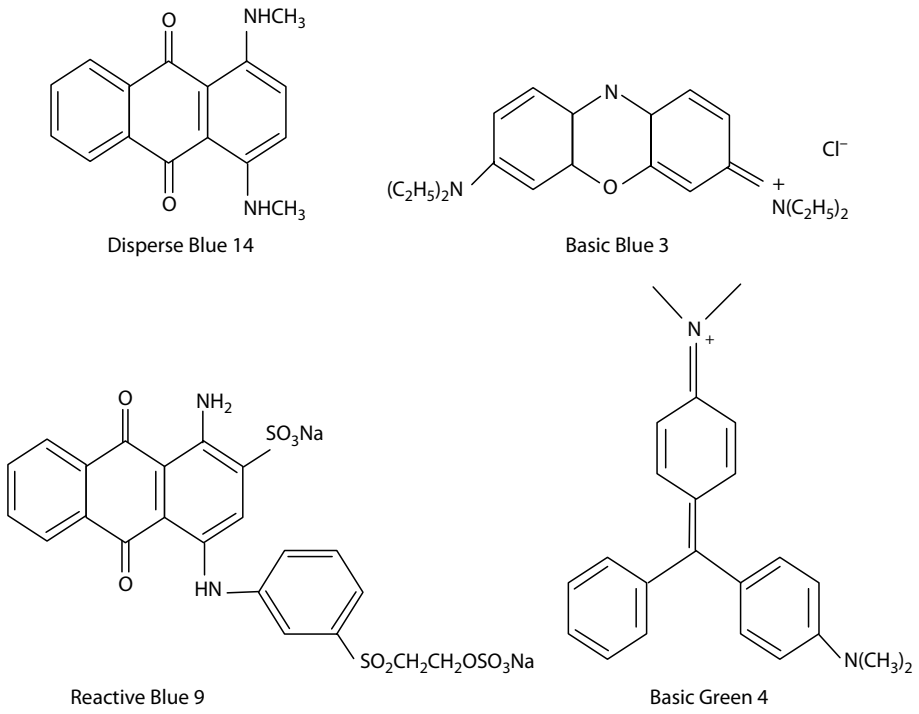

FIGURE 39.3 Reactive Black 5.

The most effective and ecological technological systems developed during the past 20 years are as a rule based on a combination of the above methods. Chitosan is a modified, natural, carbohydrate polymer derived from the chitin component of the exoskeleton of crustaceans such as shrimp, crab, crawfish, etc. Chitosan's properties like, nontoxicity, biodegradability, flocculating ability, polyelectrolisity, and possibility for regeneration lead to a number of applications. Its largest use continues to be as a nontoxic cationic flocculent in the treatment of organic polluted wastewaters and as a chelator of toxic (heavy and radioactive) metals (Knorr 1984).

Recently, different low-cost adsorbents including some industrial and agricultural wastes (McKay and Allen 1980, McKay et al. 1985, 1986, 1987, Namasivayam and Kanchana 1992, Namasivayam and Ranganathan 1992, Netpradit et al. 2003), such as fly ash, fuller's earth, waste red mud, bentonite clay, metal hydroxide sludge, peat, pith, cotton waste, rice husk, teakwood bark, etc., have been used but their effectiveness is limited and inferior to that of activated carbon. Adsorption capacity of biological materials like coir pith (Namasivayam and Kavitha 2002), banana, and orange peel (Annadurai et al. 2002) particularly toward anionic dye is very poor. Biological materials generally contain high amount of cellulose and adsorb anionic dye in a reversible process involving physical adsorption as against basic dyes where adsorption occurs there coulombic attraction and ion-exchange process (Bousher et al. 1997).

The term "biosorption" is given to adsorption processes, which use biomaterials as adsorbents (or biosorbents). Chitosan is a biopolymer and has an extremely high affinity for many classes of dyes, including disperse, direct, reactive, acid, vat, sulfur, and naphthol dyes (Figure 39.3). The rate of diffusion of dyes in chitosan is similar to that in cellulose. Only for basic dyes has chitosan a low affinity.

39.5.1 Chitin and Chitosan for Dye Removal

Mishra and Tripathy (1993) proposed a simplified classification as follows: anionic (direct, acid, and reactive dyes), cationic (basic) dyes, and nonionic (disperse) dyes. There are many structural

varieties such as acidic, disperse, basic, azo, diazo, anthraquinone colorants are the two major classes of synthetic dyes and pigments. Together, they represent about 90% of all organic colorants. It is important to note that dye molecules have many different and complicated structures, and their adsorption behavior is directly related to the chemical structure, the dimensions of the dye organic chains, and the number and positioning of the functional groups of the dyes. This is one of the most important factors influencing adsorption. However, the way adsorption is affected by the chemical structure of the dyes is not clearly identified.

One of the main problems associated with the treatment of textile dyeing plant wastewater is the removal of dyes. Discharge of dyes is objectionable not only for aesthetic reasons but also as many dyes and their degradation products are carcinogenic toward aquatic life and mutagenic for humans (Lee et al. 2003). Synthetic dyes are an important class of recalcitrant organic compounds, and are often found in the environment as a result of their wide industrial use. These industrial pollutants are common contaminants in wastewater, and are difficult to decolorize due to their complex aromatic structure and synthetic origin. They are produced on a large scale. Although the exact number of the dyes produced in the world is not known, there are estimated to be more than 100,000 commercially available dyes. Many of them are known to be toxic or carcinogenic. Generally, dyes can be classified with regard to their chemical structure (e.g., azo, anthraquinone, indigo, triphenylmethane), with regard to the method and domain of usage (e.g., direct, reactive, chromic, metal complexes, disperse, mordant, sulfur, vat, pigments), and/or with regard to their chromogen (e.g., $n \rightarrow \pi^*$, donor–acceptor, cyanine, polyenes).

Numerous low-cost adsorbents have been proposed for dye removal. Adsorption of acid dyes has been studied using peat (Poots et al. 1976a,b), activated carbon (Walker and Weatherley 1997), pith (Mckay et al. 1987, Namasivayam et al. 1998, 2001a,b, Namasivayam and Kavitha 2002), fuller's earth (McKay et al. 1985, Atun et al. 2003), and wood (Poots et al. 1976a,b, Asfour et al. 1985). Other dye-adsorbent systems have also demonstrated commercial potential, including hardwood (Asfour et al. 1985, Elgeundi 1991), pith (McKay et al. 1987), waste red mud (Namasivayam and Arasi 1997, Namasivayam and Kavitha 2002), immobilized microorganism (Chen et al. 2003), and agricultural by-products (Marshall and Champagne 1995, Marshall and Johns 1996, Namasivayam et al. 2001a,b, Valix et al. 2004, Crini 2006). Among them, nonconventional activated carbons from solid wastes, industrial by-products, agricultural solid wastes, clays, zeolites, peat, polysaccharides, and fungal or bacterial biomass deserve particular attention, as recently summarized in a review by Crini (2006).

A limited number of dye adsorption studies have been carried out on chitin and chitosan (McKay et al. 1982, Knorr 1983, McKay et al. 1983, Coughlin et al. 1990, Carlough et al. 1991, Yoshida et al. 1991, 1993, Smith et al. 1993, Stefancich et al. 1994, Safarik 1995, Shimizu et al. 1995, Kim et al. 1997, Yoshida and Takemori 1997). Several of these references relate to the adsorption of dyes on chitosan fibers or general applications to treat textile effluents. There is a lack of detailed critical analysis for the adsorption of dyes onto chitosan even though high sorption capacities have been obtained in several cases. Acid dyes are widely used in the textile industry. In this research, the ability of chitosan to remove acid dyes, namely, Acid Green 25, Acid Orange 10, Acid Orange 12, Acid Red 18, and Acid Red 73 by adsorption, has been studied.

Each has advantages and drawbacks. However, at the present time, there is no single adsorbent capable of satisfying the above requirements. Thus, there is a need for new systems to be developed. In addition, the adsorption process provides an attractive alternative treatment, especially if the adsorbent is selective and effective for removal of anionic, cationic, and nonionic dyes.

Chitosan possesses interesting characteristics that also make it an effective biosorbent for the removal of color with outstanding adsorption capacities. Compared with conventional commercial adsorbents such as commercial-activated carbons for removing dyes from solution, adsorption using chitosan-based materials as biosorbent offers several advantages (Table 39.1).

The annual worldwide production of dyes is approximated at 800,000 t and about 50% of these are azo dyes. Hence, the treatment of such wastewaters is quickly becoming a matter of great

TABLE 39.1
Advantages and Disadvantages of Chitosan and Chitosan-Based Materials Used as Biosorbent for the Removal of Dyes from Aqueous Solutions

Advantages	Disadvantages
Low-cost biopolymer with hydrophilic character	Variability in the polymer characteristics
Very abundant material and widely available in many countries, renewable resource	The performance depends on the origin and treatment of the polymer and also its degree of deacetylation
Cationic in acid pH	Not useful to remove cationic dyes
Environment friendly and biodegradable	Not much useful in the native form
Cost effective	Nonporous sorbent
Outstanding binding ability for a wide range of dyes	pH sensitivity
Fast kinetics, high selectivity in decolorizing both very dilute or concentrated solutions, versatile biosorbent	Its use in sorption columns is limited, nondestructive process

concern, and it is urgent to develop sound and cost-effective treatment technologies in order to comply with environmental regulations (Ozcan et al. 2005, Zhou and He 2007). Dye wastewaters are recognized as difficult-to-treat pollutants; they are discharged to the environment from a wide variety of sources, including textiles, printing, dyeing, dyestuff manufacturing, and food plants (Lee et al. 2008). Various physical, chemical, and biological methods are available for the treatment of dyehouse effluents; however, the color is hard to remove by conventional or advanced treatment processes. Coagulation and flocculation have their place among the conventional processes that are frequently cited for treating dye-containing effluents (Zhu et al. 2001, Allegre et al. 2004).

39.5.1.1 Coagulation/Flocculation

Conventional treatment involves a process of coagulation/flocculation. This is a versatile process, which can be used alone or combined with biological treatments, as a way of removing suspended solids and organic material, as well as promoting the extensive removal of dyes from textile industry effluents (Tunay 1996, Anjaneyulu et al. 2005). The main advantage of coagulation and flocculation is the decolorization of the waste stream by the removal of dye molecules from the dye bath effluents, and not by a partial decomposition of the dye, which can lead to even more harmful and toxic aromatic compounds (Golob et al. 2005).

Chitosan dissolved in acetic acid, proved to be very efficient for the coagulation–flocculation of Acid Black 1, Acid Violet 5, and Reactive Black 5. The charge neutralization (associated to a "bridging-like" effect) is responsible for the dye removal in acidic solutions (pH 3–4.5). It is recommended for the treatment of azo dye because of its excellent property as an environmentally friendly coagulant that can be obtained from renewable resources (Szygula et al. 2008). The coagulation–flocculation of model sulphonated azo dyes, i.e., Acid Black 1, Acid Violet, and Reactive Black 5 with the use of chitosan. Experiments were performed at three initial pH values (pH 3, 5, and 7) and four concentrations 925, 50, 100, and 200 mg L^{-1} by Szygula et al. (2008). The kinetics of the process were considered by varying the settling time, and the dye/polymer molar ratio was modified in order to determine the optimum relationship between the functional groups of the dye and the amine groups of chitosan for a maximum dye recovery. The results indicated that increasing the initial dye concentration from 25 to 600 mg L^{-1} decreases the dye adsorption/mass ratio (mol g^{-1}) whereas the pressure of the surfactant DBS and a temperature increase of 25°C–35°C increases it. The adsorption capacity of chitosan beads decreased in the presence of DBS as a result of the competitive adsorption for the same sites between the dye and surfactant molecules. The factorial experiments demonstrate the existence of a significant antagonistic interaction effect between the "concentration" and "surfactant" factors. The quantitative adsorption (N_f) modeling presents good correlations with the experimental data (Cestari et al. 2008).

39.5.1.2 Anionic or Acid Dye Removal

Colored wastewater is particularly associated with those reactive azo dyes that are used for dyeing cellulose fibers. These dyes make up approximately 30% of the total dye market (Willmott 1997, Pearce et al. 2003).

Azo dyes have been widely used as colorants in a variety of products such as textiles, paper, and leather. These chemicals present a potential human health risk as some of them have been shown to be carcinogenic (Cioni et al. 1999). It has also been shown that synthetic precursors, intermediates, by-products, and degradation products of these dyes may be potential health hazards, owing to both their toxicity and their carcinogenity. The adsorption of reactive dyes in neutral solutions using chitosan showed large adsorption capacities of 1000–1100 g kg^{-1} (Wu et al. 2000). Comparison of gas chromatography of the textile wastewater samples indicates that the nine compounds in this textile wastewater sample was significantly removed when chitosan powder or crab shell was used. The nine compounds are removed by crab shell ranges from 95% to 99% and by chitosan powder are all 99%. The adsorption capacity of crab shell powder is between 1.2×10^{-3} and 1.7×10^{-3} g and that of chitosan powder is between 2.7×10^{-3} and 4.0×10^{-3}g.

Addition of anions such as Cl^- and NO_3^- to the solution did not influence the removal of the studied dyes. The percentage of removal remained constant at a high salt concentration, tenfold exceeding the concentration of the dyes. On the other hand, the removal of AB1, AV5, and RB5 was more influenced by the SO_4^{2-} and HCO_3^- anions. In this case, the efficiency decreased from 80%–90% to 70%–80% and 30%–50% in the presence of Na_2SO_4 and $NaHCO_3$, respectively.

The removal of AB1, AV5, and RB5 was significantly lower in the presence of Na_2SO_4 and $NaHCO_3$ than in the presence of NaCl and $NaNO_3$. The impact of sulfate anions may be explained by the precipitation effect of these ions on chitosan: the interactions of the sulphate anions with protonated amine groups contributed to reducing the polymer solubility (dehydrating effect) and decreasing the availability of protonated amine groups. In the case of $NaHCO_3$, a drastic increase of the pH was observed (upto 7.1 for RB5 and upto 8.8 with AB1 and AV5). This increase of the pH entrained a significant decrease in the protonation of the amine groups of the dyes. In addition, the increase in ionic strength may affect the distribution of the charge on the chitosan molecules due to (a) a charge screening effect (Bouyer et al. 2001) and/or (b) the contraction of the thickness of the double layer surrounding the chitosan molecules. This in turn has an influence on the interaction of chitosan molecules with the dyes. In a different field, Tixier et al. investigated the effect of the ionic strength on the viscosity of an activated sludge suspension (Tixier et al. 2003), and observed that the simultaneous decrease in the particles' double layer thickness and surface charge contributed to the decrease in intraparticle interactions.

Since the deacetylated amino groups in chitosan can be protonated, the polycationic properties of the polymer can be expected to contribute to the charged interaction with anionic dyes. The adsorption of studies of various dyes on chitosan fibers, and the use of chitin, chitosan, and its derivatives has been assessed in various studies (Knorr 1983, McKay 1995, Shimizu et al. 1995, Kim et al. 1997, Juang et al. 2001, dos Anjos et al. 2002, Wong et al. 2003, Lazaridis et al. 2007, Wang and Wang 2008). Recent study of adsorption of acid Green 27 onto nanochitosan by Hu et al. (2006) showed higher capacity.

The performance of nanochitosan (with particle size range from 0.063 to 1.763 μm) as an adsorbent to remove acid dyes Acid Orange 10, Acid Orange 12, Acid Red 18, and Acid Red 73 from aqueous solution has been investigated (Cheung et al. 2009). Equilibrium isotherms have been measured and analyzed using Langmuir, Freundlich, and Redlich–Peterson equations for each individual dye. Based on the Langmuir isotherm analysis, the monolayer adsorption capacities were determined to be 1.77, 4.33, 1.37, and 2.13 mmol g^{-1} of nanochitosan for Acid Orange 10, Acid Orange 12, Acid Red 18, Acid Red 73, respectively. Removal of RB5 anions from aqueous solutions was studied using magnetic resin derived from chemically modified chitosan. The nature of interaction between the anions and the resin was found to be dependent upon the acidity of the medium. The removal

process was carried out through batch and column methods. A comparison of the adsorption capacities of the nanochitosan with those on normal chitosan was given by Wong et al. (2004).

39.5.1.3 Cationic or Basic Dye Removal

Chitosan bead was synthesized for the removal of a cationic dye malachite green (MG) from aqueous solution (Bekc et al. 2008). The kinetics of this reaction implies that sorption process obeys the pseudo-second-order kinetic model. The temperature strongly influenced the adsorption process. It is clear that the $q2$ (maximum adsorption capacity for the pseudo-second-order adsorption) values are increased by increasing the temperature. From the thermodynamic study, it is obtained that activation energy value for MG sorption onto chitosan bead is greater than 40 kJ mol^{-1}. Therefore, this type of adsorption can be considered as chemical adsorption.

39.5.2 Effect of Temperature on Dye Absorption

Adsorption of Congo red from its aqueous solution by chitosan hydrobeads has been examined at different temperatures. The results show that adsorption of the dye was maximum at 30°C. Adsorption of a solute from solution phase onto solid–liquid interface occurs by dislodging the solvent molecule (water) from interfacial region (Chatterjee et al. 2007). Moreover, at high temperature, the physical interaction between Congo red and chitosan weakened due to weakening of hydrogen bonds and van der Waals interaction. Probably with rise in temperature, the interaction between solvent and solid surface reduced exposing more number of adsorption sites (de casttro Dantas et al. 2001). This enhanced the possibility of interaction between dye and chitosan.

In addition, at elevated temperature, an increase in free volume may occur, which also may favor adsorption (Ho and McKay 1998). From 30°C onward, adsorption started to fall sharply indicating exothermic nature of the process. At 30°C, ~92.59 mg of Congo red was removed by 1 g of chitosan in the form of hydrobeads at pH 6.0, which was much higher in comparison to other adsorbent reported, such as waste wollastonite (1.21 mg g^{-1}) (Singh et al. 1984), biogas waste slurry (9.5 mg g^{-1}) (Namasivayam and Yamuna 1992), waste banana pith (20.29 mg g^{-1}) (Namasivayam et al. 1993), paddy straw (1.01 mg g^{-1}) (Deo and Ali 1993), Fe(III)/Cr(III) (44.0 mg g^{-1}) (Namasivayam et al. 1994), waste orange peal (22.44 mg g^{-1}) (Namasivayam et al. 1996), waste red mud (4.05 mg g^{-1}) (Namasivayam and Arasi 1997), activated carbon prepared from coir pith, Neem leaf powder (41.2 mg g^{-1}) (Bhattachryya and Sharma 2004), and *Aspergillus niger* biomass (14.16 mg g^{-1}) (Fu and Viraraghavan 2002).

Surprisingly, both granulated (13.80 mg g^{-1}) and powdered activated carbon (16.18 mg g^{-1}) (Fu and Viraraghavan 2002) have been found to be less efficient to chitosan. Biological materials generally contain negatively charged cellulose that repels anionic dyes by strong electrostatic repulsion resulting in poor adsorption of dye (Bousher et al. 1997). This may be one of the reasons for their low adsorption capacity toward Congo red. On the other hand, chitosan being polycationic in nature attracts Congo red, an anionic dye, and thus increases dye adsorption with dye desorption increasing with pH (Table 39.2).

TABLE 39.2
Desorption of Congo Red from Dye-Loaded Beads

pH	% of Dye Desorbed
8.0	15.4
10.0	18.0
11.0	19.0
12.0	21.2

Kinetic studies show that the adsorption reaction is pseudo-second order. Thermodynamic parameters obtained indicate that the adsorption process is an endothermic spontaneous reaction and proceeds according to Langmuir isotherm. The resin was regenerated effectively using NH_4OH/NH_4Cl buffer (pH 10).

The tabular column (Table 39.3) indicated that the adsorption process is not dependent on solution pH, since the most probable mechanism for adsorption is the interaction of the polymer quaternary ammonium groups with the dyes' sulfonate groups. In the adsorption equilibrium studies, the Langmuir equation was used to fit the experimental data

TABLE 39.3
Elution Test of Dye-Adsorbed Chitin and Modified Chitin under Caustic Conditions by Using Water and 1 M NaOH as Eluents

	pH		
Absorbent	**Initial**	**System**	**% Desorption**
1. Water elution chitin	7.96	7.63	37.09
Modified chitin	7.96	7.38	89.46
2. NaOH elution chitin	13.17	12.87	18.20
Modified chitin	13.17	12.75	3.30
3. Total elution chitin	13.17	12.75	55.29
Modified chitin	13.17	12.75	92.76

obtained, providing a maximum adsorption capacity of 1060 mg g^{-1} corresponding to 75% occupation of the adsorption sites.

39.5.3 Adsorption Behavior of Chitin and Modified Chitin

Distilled water can elute the dye from chitin and modified chitin to 37% and 89%, respectively. Accordingly, water can destroy the van der Waals force between the azo group (–N = N–), amine group ($–NH_2$), and amide group (–CO–NH–) of chitin and modified chitins. Table 39.3 indicates that modified chitin was eluted by distilled water more than chitin. This might be due to the

TABLE 39.4
Dye Removal by Chitosan-Based Adsorbents

Dye	Type	Chemical Form	qmax	Reference
Acid black 1	Anionic	Diazo	800	Guibal et al. (2003)
Acid blue 113	Anionic	Diazo	250	Guibal et al. (2003)
Acid orange 7	Anionic	Monoazo	1940	Chiou et al. (2004)
Acid green 25	Anionic	Anthraquinone	1471	Chang and Chen (2005)
Acid green 25	Anionic	Anthraquinone	850	Guibal et al. (2003)
Acid red 14	Anionic	Monoazo	1940	Chiou et al. (2004)
Acid red 73	Anionic	Diazo	728.2	Wong et al. (2004)
Acid violet 5	Anionic	Monoazo	160	Guibal et al. (2003)
Acid yellow 25	Anionic	Monoazo	220	Guibal et al. (2003)
Metanil yellow	Anionic	Monoazo	1334	Chiou and Chuang (2006)
Direct blue 14	Anionic	Diazo	90	Guibal et al. (2003)
Direct red 81	Anionic	Diazo	2383	Chiou et al. (2004)
Direct yellow 4	Anionic	Diazo	620	Guibal et al. (2003)
Mordant blue 29	Anionic	Triphenyl methane	420	Guibal et al. (2003)
Mordant brown 33	Anionic	Monoazo	2000	Guibal et al. (2003)
Reactive black 5	Anionic	Diazo	750–1000	Gibbs et al. (2004)
Reactive blue 2	Anionic	Anthraquinone	2498	Chiou et al. (2004)
Reactive red 2	Anionic	Monoazo	2422	Chiou et al. (2004)
Reactive yellow 2	Anionic	Monoazo	2436	Chiou et al. (2004)
Basic blue 9	Cationic	Thiazine	330	Chang and Juang (2004)
Basic violet 3	Cationic	Triphenyl methane	1546	Uzun and Guzel (2005)

transformation of the hydroxyl group of the primary alcohol position in modified chitin to CH_2OCl, while this position of chitin can be formulated as $CH_2–O^-$, when the leaving group (Cl) of the dye can undergo nucleophilic displacement by the hydroxyl group, so that the covalent linkage is difficult to destroy with only distilled water.

Based on the Langmuir isotherm analysis, the extremely high monolayer adsorption capacities were determined to be 645.1, 922.9, 973.33, 693.2, and 728.2 mg g^{-1} chitosan for acid green25, acid orange10, acid orange12, acid red18 and acid red73, respectively. The differences in capacities may be due to the difference in the molecular size of dye molecules and the number of sulphonate groups of each dye. Results demonstrated that monovalent and smaller dye particles have superior capacities due to increase in dye/chitosan ratio in the system, enabling a deeper penetration of dye molecules to the internal pore structure of chitosan (Table 39.4).

Muzzarelli (1973) showed that the amount of dye desorbed from the loaded chitosan beads increased with increase in pH of the eluent. Low desorption of dye with pH change indicates that physical forces such as hydrogen bonding and van der Waals force play the predominant role in the removal of Congo red by the chitosan hydrobeads because the change in pH only affects the surface charge of the adsorbent. Organic solvents, except acetone, were ineffective in desorption of Congo red from loaded beads. However, acetone desorbed the dye but the extent was only 25%.

39.6 CHITIN AND CHITOSAN FOR HEAVY METAL REMOVAL

All living beings are affected by the presence of heavy metals, which are frequently discarded in water flow near cities (Davydova 2005). The increasing level of heavy metals in the environment represents a serious threat to human health, living resources, and ecological systems (Barceloux 1999). These contaminants must be removed from wastewaters before discharge as they are considered persistent, bioaccumulative, and toxic (EPA 1998). In comparison with other industries, the electrochemical industries use less water; hence, the volume of wastewater produced is smaller, and the wastewater is highly toxic in nature because of the presence of high concentrations of metals such as copper, nickel, zinc, cadmium, and cyanide.

The use of cadmium cyanide baths in the electroplating industry generates a strong concern related to environmental impacts due to high cadmium and cyanide toxicity (Waalkes 2000). It may lead to lung inefficiency, bone lesions, cancer, and hypertension in humans (Waalkes 2000). Lead (Pb^{2+}), one of the metals of interest in this work, may be found in the environment (atmosphere, water, soil, rocks, and sediments) and in the biosphere. Lead may cause mental disturbance, retardation, and semipermanent brain damage, and it does not have an important biological activity. Another, copper (Cu^{2+}), an abundant and naturally occurring element, may be toxic if ingested in large amounts.

Numerous studies have demonstrated the effectiveness of chitosan and derived products in the uptake of metal cations such as lead, cadmium, copper, and nickel, and the uptake of oxyanions as well as complex metal ions (Muzzarelli et al. 1970, Muzzarelli and Sipos 1971, Muzzarelli and Rocchetti 1974, Sakaguchi et al. 1981, Blazquez et al. 1987, Onsoyen and Skaugrad 1990). The presence of hydroxyl and amine groups can serve as chelating sites and can be chemically modified into chitosan derivates with high adsorption capacity for ionic species (Wu et al. 2000, Chu 2002, Evans et al. 2002, Juang and Shao 2002, Guibal 2004). Cr^{6+} and Cu^{2+} adsorption onto chitosan with particular attention to the kinetics were studied by Sag and Aktay (2002).

Gyliene et al. (2006) investigated sorption capability of chitosan for Cu^{2+}-EDTA and its regeneration by electrolysis. Co^{2+} was of interest together with copper, where crab shell particles were the adsorbent (Vijayaraghavan et al. 2006). Fe^{2+} and Fe^{3+} sorption characteristics of chitosan and its cross-linked form were differentiated by Ngah et al. (2005). Chitosan modified to amine and azole resins were investigated for sorption of Hg^{2+} and UO^{2+} by Atia (2005); chitosan microspheres cross-linked with tripolyphosphate were used for the removal of both acidity and Fe^{3+} and Mn^{2+} in contaminated water in coal mining (Laus et al. 2006). Cross-linked carboxymethyl-chitosan with

Pb^{2+} as template ions was the adsorbent for lead removal in the study reported by Sun et al. (2006), and Th^{4+}-imprinted chitosan–phytalate particles were used for selective sorption of thorium by Birlik et al. (2006).

Sorption of cesium, cobalt, and europium on low-rank coal in mixture with chitosan has been investigated in a pilot study of a potential low-cost sorbent based on combined action of both active components. The application of chitosan for Mn(II) and Zn(II) ions chelation in the dual-alkali FGD wastewater was feasible. Chitosan could efficiently chelate Mn(II) and Zn(II) ions. The results obtained in this study revealed that the chitosan microsphere is an alternative for the removal of acidity, Fe(III), Al(III), and Cu(II) from coal mining wastewater.

The efficiency of adsorption of Hg^{2+} by chitosan depends upon the period of treatment, the particle size, initial concentration of Hg^{2+}, and quantity of chitosan. McKay et al. (1989) used chitosan for the removal of Cu^{2+}, Hg^{2+}, Ni^{2+}, and Zn^{2+} within the temperature range 25°C–60°C at near neutral pH. Further adsorption parameters for the removal of these metal ions were reported by Yang and Zall (1984). Maruca et al. (1982) used chitosan flakes of 0.4–4 mm for the removal of Cr^{3+} from wastewater.

Sudha et al. (2008) and Dinesh Karthik et al. (2009) reported on the removal of heavy metal cadmium and chromium from industrial wastewater using chitosan-coated coconut charcoal and chitosan impregnated polyurethane foam, respectively. Adsorption and determination of metal ions such as zinc (II) and vanadium (II) onto chitosan from seawater have been studied (Muzzarelli et al. 1970, Muzzarelli and Sipos 1971, Muzzarelli and Rocchetti 1974). Adsorption of strontium (II), cobalt (II), zinc (II), and iron (III) on chitosan from sodium chloride solution have been reported (Nishimura et al. 1995). Adsorption behavior of Cu (II) (Minamisawa et al. 1996, Wu et al. 2000) and cobalt (II) (Minamisawa et al. 1999) were investigated. The amount of cadmium removed by chitin increases with increase of these parameters at a specific time. The application to experimental results of the Langmuir and Freundlich models shows that the Langmuir model gives a better correlation coefficient.

Chitosan from different sources was used in the form of beads and flakes and the adsorption capacity for Cu(II) was almost unaffected by the physical form of the polymer while dye adsorption was much higher on beads than on the flakes. Langmuir isotherm was found to fit the adsorption process (Wu et al. 2000). Chitosan and some of its derivates have been used effectively for the adsorption of mercury(II) (Cardenas et al. 2001, Babel and Kurniawan, 2003). Radiation-cross-linked chitosan has been used recently for the treatment of wastewater contaminated with Cr(VI) (Ramnani and Sabhaewal 2006).

Much less work had been devoted for the application of chitosan and its derivatives for the absorption of radioactive elements. The sorption behavior of ^{60}Co and $^{152+154}Eu$ radionuclides onto chitosan derivative is presented. Furthermore, Pb^{2+} has also been used as a base in different types of inks, and many countries have already prohibited its use in domestic inks (Merian et al. 2004).

Studies have demonstrated that high levels of Ni^{2+} in human hair are associated to cardiovascular problems and immune dysfunction by alteration of immunoglobulin levels (Tan et al. 2004). The toxicity of Ni^{2+} has been associated to dermatitis, allergies, renal disturbances, hepatitis, infertility, lung cancer, stomatitis, gingivitis, insomnia, and nauseas. These problems are prevented in humans (Tan et al. 2004) by appropriate levels of Fe^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+}, which inhibit the absorption and retention of Ni^{2+}.

Kawamura et al. (1993) prepared a porous polyaminated chitosan-chelating resin by cross-linking poly (ethylene amine) onto chitosan beads. The resultant beads showed high capacity and high selectivity for the adsorption of metal ions. Chang and Chen (2005) investigated the adsorption of copper on magnetic nanochitosan beads. They found that the chitosan-bound Fe_3O_4 nanoparticles were efficient for the removal of Cu(II) ions at pH > 2 with a maximum adsorption capacity of 21.5 mg g^{-1} (Table 39.5).

Li and Bai (2005) investigated a new amine-shielding method for cross-linking chitosan with EGDE to improve the chemical stability of beads in acidic conditions. Hasan et al. (2003) noted

TABLE 39.5
Adsorption Capacity of Various Forms of Chitosan for Heavy Metal Copper

Adsorbent	pH	Adsorption Capacity (mg g^{-1})	Reference
Chitosan	5.6	93.4	Ni and Xu (1996)
Chitosan	5.0	16.2	Huang et al. (1996)
Chitosan-based resin	5.6	131.2	Ni and Xu (1996)
Amidoxine chitosan resin	5.0	93.4	Kondo et al. (1999)
Amidoxine chitosan resin	5.0	93.4	Kondo et al. (1999)
Imprinted chitosan resin	5.0	174	Juan et al. (1999)
Chitosan (shrimp)	5.5	152.7	Tseng et al. (1999)
Chitosan (crab)	5.5	168.2	Tseng et al. (1999)
Chitosan (lobster)	5.5	165.2	Tseng et al. (1999)
Chitosan (cuttlebone)	5.5	235.9	Tseng et al. (1999)
Chitosan	5.0	160	Wu et al. (1999)
Chitosan (shrimp)	5.0	300	Wu et al. (2000)
Chitosan	5.0	80	Schmuhl et al. (2001)
Chitosan-coated perlite bead	4.5	104	Shameem Hasan et al. (2008)
Chitosan (perlite basis)	4.5	325	Shameem Hasan et al. (2008)
Chitosan diacetate crown ether (CCTS–2)	4.1	31.3	Srinivasa Rao et al. (2009)

that by dispersing chitosan on an inert substrate, perlite, its adsorption capacity for Cr(VI) could be enhanced. Kalyani et al. (2005) used chitosan-coated perlite beads to absorb copper and nickel from aqueous solution. It is assumed that the active group, such as NH_2, becomes more readily available when chitosan is dispersed on perlite. Arsenic is known to be a poison of acute toxicity and has long-term carcinogenic properties, particularly with respect to lung and skin cancer (Desesso et al. 1998). The European Commission (EC 1998), the US Environmental Protection Agency, and the World Health Organization (WHO 2003) have already revised the maximum concentration limit for arsenic in drinking water by decreasing it from 50 to 10 μg L^{-1}.

39.7 CHITOSAN FOR GENERAL WASTEWATER TREATMENT

Fouling is a problem encountered with most filtration processes as it reduces productivity and impairs the capabilities of the membrane due to the decline in flux because of concentration polarization or fouling of the membrane (Fane and Fell 1987). Feasibility of using chitosan as an antifouling agent in a bioreactor to help decrease fouling was successfully demonstrated by Le Roux et al. (2005).

One potential application of chitosan complexes in seafood processing is the treatment of surimi wash water (SWW), which contains 0.1%–2.3% protein (Morrissey et al. 2000) to obtain not only clean wash water for reuse in the plant but also to recover suspended proteins for use in feed production. Flocculation of SWW protein by using Chi–Alg at a concentration of 100 mg complex/L SWW for 1 h achieved high protein adsorption and turbidity reduction (Wibowo et al. 2005a,b). The Chi–Alg complex is an effective alternative in not only recovering soluble proteins that would otherwise be discarded into the environment, but more so an economically viable downstream process over expensive, commercial membrane treatments and their limited use due to periodic fouling (Savant 2001). Insoluble proteins can be recovered in the same step used for SWW (Wibowo et al. 2007).

Fluoride normally enters the environment and human body through water, food, industrial exposure, drugs, cosmetics, etc. However, drinking water is the single major source of daily intake (Sarala and Rao 1993). The World Health Organization has specified the tolerance limit of fluoride content of drinking water as 1.5 mg L^{-1} (WHO 1984). The presence of a large amount of fluoride induces dental and skeletal fluorosis and nonskeletal fluorosis. The only option to prevent and control fluorosis

is the defluoridation of drinking water. Lanthanum chitosan adsorbents show excellent removal of fluoride from water, which is much better than bare chitosan and chitin (Kamble et al. 2007).

39.8 CHITOSAN ULTRAFILTRATION MEMBRANE FOR METAL REMOVAL

In the past 20 years, UF has been shown to be promising for the removal of trace metals from aqueous streams, provided that these metals were primarily bound to water-soluble polymers (Chaufer and Deratani 1988). Juang et al. had worked to examine the removal efficiency of divalent metals including Cu(II), Co(II), Ni(II), and Zn(II) from dilute solutions with chitosan-enhanced UF (Juang et al. 2000). The use of chitosan to enhance UF removal of Cu(II), Co(II), Ni(II), and Zn(II) from dilute solutions has been investigated. It is shown that the pH plays the most important role on UF flux and metal rejection in the presence or absence of chitosan. The binding between metals and the amino groups of chitosan is the type of coordination. The removal of Cu(II) is more easier than other metals due to its higher coordination ability with chitosan.

39.9 CONCLUSION

Environmental requirements are assuming great importance, since there is an increased interest in the industrial use of renewable resources such as starch and chitin. Considerable efforts are now being made in the research and development of polysaccharide derivatives as the basic materials for new applications. In particular, the increasing cost of conventional adsorbents undoubtedly makes chitin and chitosan-based materials one of the most attractive biosorbent for wastewater treatment. Chitin and chitosan biopolymers have demonstrated outstanding removal capabilities for certain pollutants such as dyes and metal ions as compared to other low-cost sorbents and commercial-activated carbons. Biopolymer adsorbents are efficient and can be used for the decontamination of effluents (removal of pollutants) and for separation processes (recovery of valuable metals).

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Part VII

Agricultural and Biotechnology Applications of Chitin and Chitosan and Their Derivatives

40 Chitin, Chitosan Derivatives Induce the Production of Secondary Metabolites and Plant Development through *In Vitro* and *In Vivo* Techniques

Abdul Bakrudeen Ali Ahmed and Se-Kwon Kim

CONTENTS

40.1 INTRODUCTION

Chitin is an important component in shells of arthropods and fungi, and is widely used as an eliciting agent of cell defense reaction in plants (Shibuya and Minami 2001). It has been one of the best economic value products due to its versatile biological activities and chemical applications in medical (Yadav and Bhise 2004) and pharmaceutical (Kato et al. 2003) areas. Chitin and chitosan have shown peculiar properties such as biodegradability, biocompatibility, bioactivity, selective permeability, polieletrolic action, chelation, ion exchange properties, antitumor and antimicrobial activity (Yadav and Bhise 2004), absorption capacity (Franco et al. 2004), and biopolymer studies (Campos-Takaki and Galba 2005).

Chitin is a major cell wall constituent of higher fungi belonging to chitridiomycetes, ascomycetes, basidiomycetes, deuteromycetes, insect exoskeletons, and crustacean shells (Felse and Panda 1999). Chitosan is a polysaccharide derived from a low acetyl form of chitin, mainly composed of glucosamine and *N*-acetyl glucosamine, which are the constituents of both cellulose and chitin. It has a strong positive charge, and attracts negatively charged molecules (Freepons 1991). Chitinase is a hydrolytic enzyme that specifically degrades chitin and has gained much attention worldwide (Wang et al. 2006). It was produced by several bacteria (Ajit et al. 2006), fungi (Akagi et al. 2006, Viterbo et al. 2001), and higher plants (Troedsson et al. 2005). In this aspect, chition is one of the major resources for the production of chitinase, and the resistance characterized in plants, pathogens. It shows biological control of various fungal pathogens (Chang et al. 2007, de la Vega et al. 2006). Microorganisms produce the chitinase primarily for the assimilation of chitin as the carbon and nitrogen source (Kupiec and Chet 1998). The filamentous fungi have been considered an attractive source of chitin and chitosan for industrial applications because their specific products can be manufactured under standardized conditions (Nemtsev et al. 2004). This chapter deals with the chitin and chitosan derivatives, which improves the production of various secondary metabolites under *in vitro*, *in vivo* conditions and their biotechnological applications in detail.

40.2 CHALLENGES IN DRUG DISCOVERY

Plants have been utilized as medicines for thousands of years for curing various ailments and diseases (Samuelsson 2004). These medicines were initially used in the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations. In the recent history, use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early nineteenth century (Kinghorn 2001, Samuelsson 2004) followed by other drugs such as cocaine, codeine, gymnemic acid, digitoxin, and quinine (Ahmed et al. 2009, Samuelsson 2004). Isolation and characterization of pharmacologically active compounds from medicinal plants continue today. More recently, drug discovery techniques have been applied to the standardization of herbal medicines to elucidate analytical marker compounds (Balunas and Kinghorn 2005). Among them are alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids, and inorganic ions used as a marker (Gurib-Fakim 2006).

Despite the successful evidence of drug discovery from medicinal plants, future endeavors face many challenges. Pharmacognosists, phytochemists, and other natural product scientists will need to continuously improve the quality and quantity of compounds that enter the drug development phase to keep pace with other drug discovery efforts (Butler 2004). This process of drug discovery has been estimated to take an average of 10 years upward (Reichert 2003) and cost more than $800 million (Dickson and Gagnon 2004). Much of this time and money is spent on the numerous leads that are discarded during the drug discovery process. In fact, it has been estimated that only 1 in 5000 lead compounds will successfully advance through clinical trials and be approved for use. Lead optimization and identification (involving medicinal and combinatorial chemistry), lead development (including pharmacology, toxicology, pharmacokinetics, ADME [absorption, distribution, metabolism, and excretion], and drug delivery), and clinical trials all will take the considerable length of time. Drug discovery from medicinal plants has traditionally been lengthier and more complicated than other drug discovery methods. As such, many pharmaceutical companies have eliminated or scaled down their natural product research (Balunas and Kinghorn 2005).

40.2.1 Natural Products

Natural product chemistry is a multidisciplinary field that combines chemistry, botany, biochemistry, environmental science, medicine, etc. With the development of science and technology, this field has gained increasing importance in various health aspects of mankind. History tells us that

mankind was interested in naturally occurring substances. In ancient cultures, plant extracts were utilized as healing substances and tribal hunters, and the use of plant extracts as healing substances is prevalent today. The majority of natural products are isolated from plant origin. It is mainly due to the ease of the isolation process. Natural products are usually given a trivial name derived from the plant origin. Recent developments in biology have given some hints about the properties of these compounds:

- Many natural products have a regulatory role.
- Some act as the chemical policeman against a pest.
- They function as chemical communicators (or) messengers.
- They behave as chemicals for protection.

40.2.2 Strategies for Increasing the Secondary Metabolites Production

Plant tissue cultures are potentially valuable for studying the biosynthesis of secondary metabolites, and may eventually provide an efficient way of producing commercially important plant metabolites. In spite of these obviously desirable features, the use of tissue culture has so far been very limited, because many cultures do not produce significant secondary products *in vitro* (Butcher 1992).

Plant cell cultures are an attractive alternative way to produce high-value secondary metabolites when compared to the whole plant (Ravishankar et al. 1999). Plant cells are biosynthetically totipotent, which means that each cell in culture retains complete genetic information, and hence it can produce the range of chemicals found in the parent plant.

In the past two decades, plant cell biotechnology has evolved as a promising new area within the field of biotechnology, focusing on the production of plant secondary metabolites. The production of these compounds is often low (less than 1% dry weight) and dependent greatly on the physiological and developmental stage of the plant. Many secondary metabolites have a complex and unique structure and their production is often enhanced by both biotic and abiotic stress conditions (Dixon 2001).

40.2.3 Primary and Secondary Metabolites

Plant cells produce far more chemical compounds than is necessary for their basic functions i.e., biochemical pathways for survival and propagation. Basic or primary metabolism refers to all biochemical processes for the normal anabolic and catabolic pathways, which result in assimilation, respiration, transport, and differentiation. Basic or "primary" metabolism is shared by all cells, while "secondary metabolism" generates diverse and seemingly less essential or nonessential by-products called "secondary products." The secondary products are the colors, flavors, and smells, which are the sources of fine chemicals such as drugs, insecticides, dyes, flavors, and fragrances, and plant-growth regulators found in medicinal plants.

While primary metabolism consists of biochemical pathways that are in general common to all cells, secondary metabolism consists of a large number of diverse processes that are specific to certain cell types. Plant pigments, alkaloids, isoprenoids, terpenes, and waxes are some examples of secondary products. The role of many of the secondary products has been rather ambiguous, and initially they were thought to be just waste materials. However, considering their nonmotile nature and the lack of the sophisticated immune system that we have, plants have to develop their own defense system against pathogens and predators, and systems to lure motile creatures for fertilization and dissemination. Indeed, many of the secondary products are bactericidal, repellent (by bad tastes, etc.), or even poisonous to pests and herbivores.

Plant secondary metabolites represent a tremendous resource for scientific and clinical researches and new drug development. Overall, their pharmacological value not only remains undiminished until today, but is increasing due to constant discoveries of their potential roles in healthcare and as lead chemicals for new drug development.

40.3 CHITIN AND CHITOSAN IN PLANT BIOTECHNOLOGY

Water soluble chitin 50 and chitosan were developed from the chitin and has been used as exogenous elicitors for the production of chitinases and anthraquinone in *Morinda citrifolia* thorugh cell culture. On the other hand, the commercial pectin derived from *M. citrifolia*, it can be induced chitinase and lysosome activities (Heike and Dietrich 1994).

40.3.1 *In Vitro* Conditions—Secondary Metabolites Production

40.3.1.1 Elicitors (or) Precursors

Chitosan induces the phytoalexins' secondary metabolites accumulation by antifungal responses and enhances protection from further infections (Vasyukova et al. 2001). Besides, chitosan and chitin oligomers have increased the activities of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL); these are key enzymes of the phenyl propanoid pathway, in soybean leaves (Khan 2003), and sweet basil (*Ocimum basilicum* L.) (Kim et al. 2005). PAL and TAL are modified via the phenyl propanoid pathways to produce secondary metabolites, including lignin, flavonoids pigments, and phytoalexins, which play an important role in plant–pathogen interactions. Sometimes, chitosan increases the polyphenol oxidase (PPO) activity in disease-resistant cultivars (Raj et al. 2006). However, oxidation of phenolic compounds associated with enhanced resistance to pathogens may involve PPO, which could generate reactive oxygen species (ROS) (Mayer 2006). Suspension-cultured wheat cells in a growth medium with chitin or chitosan led to a strong increase in the extracellular peroxidase activity in *Pantoea agglomerans* (Ortmann and Moerschbacher 2006).

Recently, Cho et al. (2008) reported that methyl jasmonate (MJ) and yeast extract (YE) was induced the protein expression and benzophenanthridine alkaloid accumulation in *Eschscholtzia californica* suspension cell cultures. These results were confirmed that the dihydrobenzophenanthridine oxidase (DHBO) is induced by YE and not by MJ. YE and chitin had similar effects on sanguinarine production and DHBO expression.

Sometimes explant has been one of the important factor for secondary metabolites production, which could be economicallly feasible and the protective of the plant (Figure 40.1). This study was done in different hours such as 6, 12, 24, 48, 72, and 168 h with different concentrations of chitosan (used by an elicitor). A positive effect on the production was exerted in particular by elicitation of

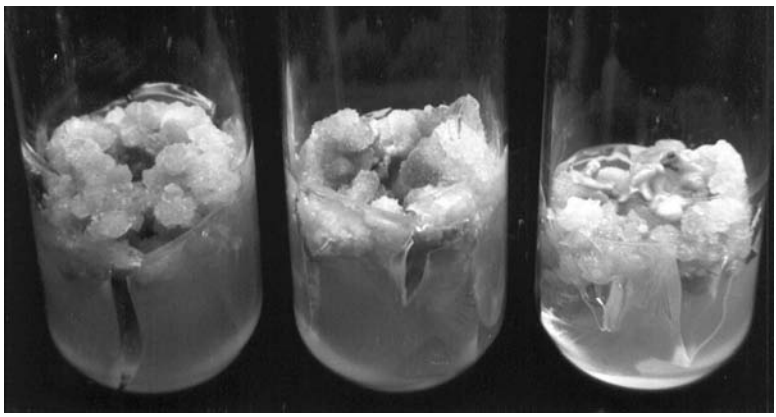

FIGURE 40.1 Secondary metabolites production under the callus culture. (From Ahmed, A.B.A. et al., *In vitro* production of gymnemic acid from *Gymnema sylvestre* (Retz) R.Br. Ex. Roemer & Schultes through callus culture under stress conditions, In: Jain, S.K. and Saxena, P.K. (eds.), *Protocols for In Vitro Cultures and Secondary Metabolites Analysis of Aromatic and Medicinal Plants, Serious: Methods in Molecular Biology*, Vol. 547, Humana Press Inc., Totowa, NJ, 2009, p. 93. With permission.)

the suspension culture. The maximal content of anthracene derivatives is found by photometric determination after 24 h treatment of chitosan (Kasparova and Siatka 2001).

Amaranthus tricolor plant cells were entrapped with chitosan gel to determine the polycationic properties of chitosan on plant cell membrane permeability. On the fifth day, maximum tricolor cells were released from chitosan-immobilized cells (Knorr and Teutonico 1986).

Biosynthesis of the flavonolignan silymarin, a constitutive compound from the fruit of *Silybum marianum* with strong antihepatotoxic and hepatoprotective activities, is severely reduced in cell cultures of this species. It is well known that elicitation is one of the strategies employed to increase accumulation of secondary metabolites. YEs, chitin, and chitosan were compared with respect to their effects on silyamarin accumulation in *S. marianum* suspensions with YE–stimulated production (Sanchez-Sampedro et al. 2005).

40.3.1.2 Microorganisms

In vitro methods of chitin degradation by bacteria such as *Bacillus laterosporus, Bacillus pabuli, Bacillus stearothermophiilus, Bacillus subtilis, Bacillus thuringiensis* sub sp. play a specific role in secondary metabolite production with suitable culture medium (YNB, LBB, NB) (Soiuza et al. 2005). Although 0.3% colloidal chitin concentrations significantly enhanced the chitinase activity in *Fusarium chlamydosporum* (Mathivanan et al. 1998), the additional colloidal chitin at 0.5% induced the maximum chitinase production in *Bacillus* sp. NCTV2 (Wen et al. 2002), *Alternaria alternata* (Sharaf 2005), and *Trichoderma harzianum* (Sandhya et al. 2005).

Since 1902, the plant cell tissue culture has been used to study biosynthetic pathways and large-scale production of secondary metabolites production by plant cells (Verpoorte et al. 2002). Even though, chitin was used as an elicitor in citrus plant culture for increases the PAL and peroxidase activities in *C. auranitium* cells. Unfortunately, the enzyme activity was decreased and produced the non-lignified cells in citrus plant culture. (Gallao et al. 2007). Chitin induced a fast (2 h) defense response in *C. aurantium* suspension cells and restored their normal development after 6 h. The results obtained show that lignification with peroxidase and PAL activity was increased and morphological changes were similar to cell aging and death for this dicot plant by chitin treatment Figure 40.2 (Gallao et al. 2007).

Allosamidin, one of the typical secondary metabolites, is isolated from streptomyces using chitinase inhibitors. It can promote the production of chitinase detected in the culture by filtrate, and chitin hydrolytic activity was inhibited by allosamidin. These results suggest that allosamidin acts as a key signal molecule for chitinase production, which may be useful for its growth in the chitin-rich environment (Suzuki et al. 2006).

Chrysosporium lobatum, one of the biological control agents for mosquitoes, also has secondary metabolites and larvicidal properties. The secondary metabolites were released in sabouraud's dextrose broth (SDB) and chitin broth (CB) medium on *C. lobatum* (Mohanty and Prakash 2009). This precipitate of secondary metabolites was effective to both the mosquitoes, and the insectidal effect was reduced after exposure for 5 min at 120°C (Mohanty and Prakash 2009).

40.3.1.3 Plant Cell Culture

The cell cultures have the following advantages: (1) the possibility of keeping the cells active and stable over a long period of time, (2) protection of sensitive cells against hydrodynamic stress, (3) increased reaction rate and productivity, and (4) the possibilities for repeated use of the immobilized cells and for continuous operation. When fragmented, chitin (β-1,4-*N*-acetylglucosamine oligomers) can elicit these defenses, including phytoalexin production (Ramonell et al. 2005), localized deposition of lignin, and an increase in peroxidase, L-phenylalanine ammonia-lyase, and glucanase activities (Boudet et al. 1995). Xyloglucan oligosaccharides (Warneck et al. 1996) and chitosan (Peltonen et al. 1997) are other saccharides used as elicitors.

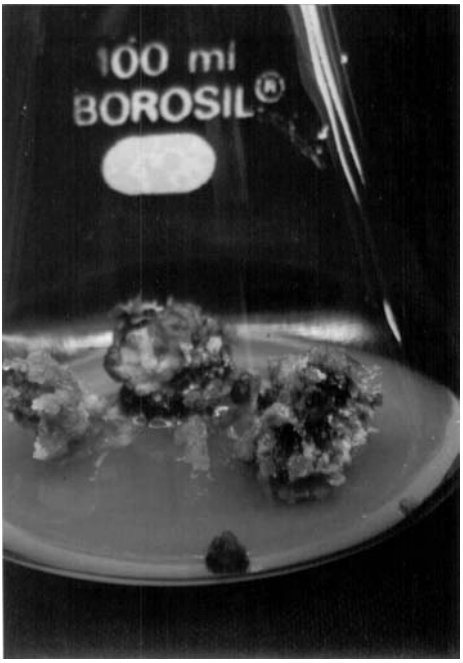

FIGURE 40.2 Secondary metabolites morphological changes in chitin treatment. (From Ahmed, A.B.A. et al., *In vitro* production of gymnemic acid from *Gymnema sylvestre* (Retz) R.Br. Ex. Roemer & Schultes through callus culture under stress conditions, In: Jain, S.K. and Saxena, P.K. (eds.), *Protocols for In Vitro Cultures and Secondary Metabolites Analysis of Aromatic and Medicinal Plants, Serious: Methods in Molecular Biology*, Vol. 547, Humana Press Inc., Totowa, NJ, 2009, p. 93. With permission.)

Sometimes, the plant cells release useful metabolites into the culture broth, and efficient production of useful metabolites in the cells can be achieved by using an immobilized cell culture method (Shuler 1981). The biosynthetic pathway of plumbagin has been studied (Durand and Zenk 1974), including the signal molecules (chitin elicitors) that trigger the formation of this product. Polysaccharides, rich in galacturonic acid, from the primary cell walls of plants also serve as signal molecules that trigger the defense responses of plant; for this reason, a purified apple pectin is used as an elicitor (Darwil and Albersheim 1984).

The use of biotic or abiotic elicitors is one way to increase the secondary metabolites in the *in vitro* cultures (Bohlmann and Eilert 1994). Supplementation of hairy root cultures of *Brugmansia candida* with chitosan at certain concentrations was found to increase the content of scopolamine, hyoscyamine, and tropane alkaloids, which are valuable anticholinergic drugs employed as antispasmodics and in the treatment of motion sickness (Yamada et al. 1994). Besides, hairy root cultures of *Hyoscyamus muticus* supplemented with chitosan produced fivefold hyoscyamine than the control (Sevon et al. 1992). Many researchers suggest that the chitosan added to suspension cell cultures of parsley (*Petroselinum crispum*) elicited a rapid deposition of the β-1,3 glucan, cellulose in the cell walls and a slower formation of coumarins (Conrath et al. 1989). The addition of chitosan to the culture media has been shown to enhance the production of hernandulcin, a minor constituent (0.004% dry wt) of the essential oil obtained from the aerial parts of *Lippia dulcis* Trev (Sauerwein et al. 1991). Treating hairy root cultures of *Trigonella foenum-graecum* with 40 mg/L chitosan increased threefold of diosgenin, a spirostanol important for the synthesis of steroid hormones (Merkli et al. 1997).

N-acetylchitosaccharides presence was varied depending on the chemo/physiological conditions of the cells, whereas the addition of the chitin growth media (Gamborg et al. 1968) caused much higher root than in a production media (Heller 1953). However, shinkonin production (a naphthoquinone structurally similar to plumbagin) by *Lithospermum erytrorbizon* in suspension cultures

was augmented by commercial citrus polygalacturonides (Fukui et al. 1990). However, shinkonin production (a naphthoquinone structurally similar to plumbagin) by *Lithospermum erytrobizon* in suspension cultures was augmented by commercial citrus polygalacturonides (Fukui et al. 1990). Besides, commercial chitin with *Botrytis* fungal was used for the production of sanguinarine in *Papaver somniferum* by suspension culture (Wiliams et al. 1992). However, plumbagin production was a decreased in addition to the chitin/CM-chitin (Nahalka et al. 2004).

40.3.1.4 Bioreactor

The continuous production of secondary metabolites was designed based on plant suspension culture with suitable bioreactor by the use of *Eschscholzia californica*. The secondary metabolite cells were elicited with chitin at fourth day in liquid culture media, these free secondary metabolite cells were debris and continuously pumped into the extraction columns containing fluidized XAD-7 resins. The production level was similar to the culture without resins with a maximum of 2.06 µmole/g DW total alkaloids, with 1.54 µmole/g DW of resins by perfusion cultures. However, the nutritional status was identified by elicitation as a major cause and reduced the production (Dobbeleer et al. 2006).

Griseofulvin, one of the secondary metabolites, was produced from fungal species with suitable morphology of solid-state fermentation (SSF). Griseofulvin, one of the secondary metabolite was produced from fungal by solid-state fermentation (SSF). This fermentation media contains 50% (v/w) initial moisture, and then added 1 mL suspension (10^6 spores/mL) under agitation at 250 rpm. In addition, 0.1% choline chloride precursor was significantly produced the griseofulvin after 9th day; the complete fermentation experiment was done in 28th days. The biomass was estimated by the chitin method (Saykhedkar and Singhal 2004).

Flavonolignan silymarin, one of the secondary metabolites compounds, is isolated from fruits of *S. marianum* with strong antihepatotoxic and hepatoprotective activities. Yeast-extracted (YE) chitin and chitosan-containing medium were compared with silymarin accumulation in *S. marianum* suspension culture. This study shows that YE promotes the flavonolignan silymarin production in *S. marianum* (Sanchez-Sampedro et al. 2005).

Arabinogalactans are secondary metabolites isolated from immobilized callus cultures of *Tinospora cardifolia*. These plant cells were immobilized in calcium chloride, sodium alginate with chitosan solution, and the cells could be permeabilized with polymeric neutral resin were used for trouble-free secondary metabolites production. The research findings show that the resin and chitosan additions increased the production of arabinogalactan 10 fold compared with controls (D'Souza 2005).

Chitin and chitosan were grown in Yam bean culture media and produced higher yields of biomass (24.3 g/mL) in 96 h from *Cunninghamella elegans* (UCP 542). The high-level chitosan (66 mg/g) and chitin (440 mg/g) were produced at 48 and 72 h. This result suggests that high biotechnological potential of yam bean (as an economic source) can be used to produce chitin and chitosan by *C. elegans* through the fermentation process. The new medium using yam bean for production of the chitin and chitosan may be used for many purposes to reduce the cost of the fermentation process (Christina et al. 2007).

Advanced technology of a benchtop bioreactor has facilitated continuous extraction of secondary metabolites, which is designed for *Catharanthus roseus* L. (G.) and *Santalum album* in plant cell suspension cultures. *C. roseus* cells were exposed by biotic elicitors (*Aspergillus niger*, crude chitin), abiotic elicitors (mannitol, MJ), and induced the alkaloid production (Valluri 2009).

40.3.1.5 Micro Propagation (Orchid Production)

Chitosan can be used as a plant-growth enhancer for orchid production, especially for immature plants through plant tissue culture, and has increased the length of the stalks of *Dendrobium* "Missteen." Possibly, chitosan may give the signal to the gibberellins. Chitosan has been enhancing the growth and development by signaling pathway, and is related to an auxin biosynthesis via a tryptophan-independent pathway. However, chitosan has inconsistent effects on growth and

development of mature orchid plants. Sometimes, chitosan can reduce disease severity in orchid and increase the PAL and PPO activity, the lignification resulting from increased biosynthesis of phenolic compounds, induced secondary metabolites, and systemic acquired resistance (SAR) (Uthairatanakij et al. 2007).

Chitin and chitosan are used as elicitors for inducing phytoalexin accumulation in plant tissue culture, and they can be used as tools for enhancing secondary metabolite yields such as coumarins, furanocoumarins, acridone, quinolone alkaloids, and flavonoids. These are the secondary metabolites that increased significantly in shoots of *Ruta graveolens* by the addition of chitin or chitosan (Orlita et al. 2008). The addition of 0.01% chitin caused an increase in the quantity (mg/g dry weight) of coumarins (pinnarin upto 116.7, rutacultin up to 287.0, bergapten up to 904.3, isopimpinelin up to 490.0, psoralen upto 522.2, xanhotoxin upto 1531.5, and rutamarin upto 133.7). The higher concentration of chitosan (0.1%) induced the simple coumarins (pinnarin up to 116.7, rutacultin up to 287.0, furanocoumarins (bergapten up to 904.3, isopimpinelin up to 490.0, psoralen up to 522.2, xanhotoxin upto 1531.5) and dihydrofuranocoumarins (chalepin up to 18 and rutamarin up to 133.7). These are secondary metabolites produced by the treatment of chitin and chitosan with natural resistance mechanism of *R. graveolens*. Even though, chitin and chitosan containing media was significantly increased the secondary metabolites such as xanthotoxin, isopimpinelin, psoralen, chalepin or methoxulate dictamine derivatives (Orlita et al. 2008).

Oat plant produced a group of secondary metabolites termed avenanthramides (avn), and has been biosynthesized by the enzyme action of hydroxycinnamoyl COA: hydroxyanthranilate *N*-hydroxycinnamoyl transferase (HHT), which catalyzes the condensation of several cinnamate COA. Suspension cultures also produce large quantities of avnA and G by chitin elicitation. These findings suggest that the utility of oat suspension culture as a tool for more detailed investigation of the mechanisms trigger their biosynthesis as well as the factors dictating the particular types of avenanthramides biosynthesis (Wise et al. 2009).

40.3.2 *In Vivo* Conditions—Secondary Metabolites Production and Plant Development

Chitin and chitosan were used against the plant-affecting pathogens and insects, and it could be possible to enhance a wide variety of useful metabolites (Cisneros-Zevallos 2003). Some of these metabolites have the light absorptive properties, harvest light for photosynthesis, and protect the cells from damaging effects of high-energy radiation while others promote defensive action against herbivores and pathogens (Harborne and Williams 2000).

Chitosan enhances phytoalexin secondary metabolites in germinating peanut (Cuero et al. 1991), and also induces the formation of phytoalexins in legumes and solanaceous plants (Cote and Hahn 1994). Thus, chitosan may be involved in the signaling pathway for the biosynthesis of phenolic compounds and has been inducing chitinase and chitosanase, which are members of a group of plant pathogenesis-related (PR) proteins (van Loon et al. 1994). These PR proteins can degrade the cell walls of some phytopathogens and, consequently, may play a role in host plant defense systems (Graham and Sticklen 1994). Moreover, chitosan may also induce plant immune systems (SAR), which is long lasting and often confers broad-based resistance to the different pathogens. SAR develops in uninfected parts of the plant; as a result, the entire plant is more resistant to a secondary infection such as antifungal chitinases, β-1,3 glucanases, PR-1, and PR-5 (Sathiyaba and Balasubramanian 1998). Further studies have shown that chitosan induces the expression of various genes involved in plant-defense responses such as a gene encoding PAL and protease inhibitors (Vander et al. 1998). Even though chitosan has been shown to elicit defense genes in several species such as rice (Rakwal et al. 2002), slash pine (Mason and Davis 1997), and tomato (Ben-Shalom et al. 2000). These genetic studies support that chitosan may improve jasmonic acid (JA) pathways, since transcription activation of genes encoding PAL and protease inhibitors were induced by both JA and chitosan (Doares et al. 1995). *Arabidopsis* crude plant extracts with chitin under suitable

conditions produced *N*-acetylglucosamine and secondary metabolites (Mitchell-Olds and Pedersen 1998). *Arabidopsis* crude plant extracts with chitin under suitable conditions produced *N*-acetyl glucosamine and secondary metabolites (Mitchell-Olds and Pedersen 1998). But, the chitin derivatives of chito-oligomers were involved in the defense mechanism of the plants (Ramonell et al. 2004); and has inducing the gene expression and reactive oxygen in plants (Ramonell et al. 2005).

40.3.2.1 Microorganisms

Chitin-degrading bacteria (*B. laterosporus*), *B. pabuli* (Frandberg and Schnurer 1994), *B. stearothermophiilus* (Sakai et al. 1994), *B. subtilis* (Wang et al. 2006), and *B. thuringiensis* sub sp. (de la Vega et al. 2006) were isolated from agricultural soil fields, and they have aided the production of chitinase. El-Tarabily et al. (2000) reported 39 different chitinolytic bacteria were isolated from rhizosphere soils as already demonstrated the presence of more number of chitin degrading bacteria in agricultural fields. The presence of chitinlytic bacteria in the crop rhizosphere soils is highly beneficial as they could suppress the plant pathogenic fungi near the root zone and provide sustainable plant protection against root diseases.

40.3.2.2 Horticulture Development

Chitosan and its derivatives have increased the growth rates of roots and shoots of daikon radish (*Raphanus sativus* L.) (Tsugita et al. 1993), number of flowers in bushes (Utsunomiya and Kinai 1994), flower-stem length, development of leaves (Wanichpongpan et al. 2001), promoted vegetative growth, and enhanced the flower buds in lisianthus (*Eustoma grandiflorum*) (Uddin et al. 2004). Chitosan also promoted the growth of various crops such as cabbage (*Brassica oleraea* L. var. 'Capitata') (Hirano 1988), soybean sprouts (Lee et al. 2005), and sweet basil (Kim 2005). Chitogel, a derivative of chitosan, was found to improve vegetative growth of grapevine plantlets (Ait Barka et al. 2004). Experiments were also conducted in various crops such as chili, Chinese cabbage, celery, bitter cucumber, and rice (Boonlertnirun et al. 2005). This finding suggests that chitosan significantly increased growth rates of chili and the yield of Chinese cabbage, but had no effect on rice (Chandrkrachang et al. 2003). Lee et al. (1999) found that chitosan treatment increased the yield of soybean sprouts. However, the mechanism of action of chitosan on plant growth remains unclear.

M-acetyl glucosamine chitin oligosaccharides (NA-COSs) play a crucial role in plant biotechnology, secondary metabolites production, plant resistance (chitin in cell walls), and symbiotic bacteria released in root nodules for nitrogen fixation (Asaoka 1996). It has an establishment of a host-specific symbiosis between legumes, and their rhizobia were determined in plants (Cohn et al. 1998).

Chitin oligosaccharides (NA-COSs) were elicitated from different plant species, being 6–10 h for *Oryza sativa* (Ren and West 1992), 40 h for *Vanilla planifolia* (Funk and Brodelius 1990). The detection of coniferyl aldehyde, emphasized by the red color, after the phloroglucinol reaction supports the hypothesis of deposition of phenolic compounds in the cell walls treated with chitin (Vallet et al. 1996). The increase in the POD activity of *C. aurantium* cells treated with chitin fragments indicates that these fragments are capable of inducing a stress response in these cells. As many authors have observed, chitin has the capacity to elicit suspension-cultured cells from species such as slash pine (Lesney 1990), potato (Dornegburg and Knorr 1997), and wheat (Vander et al. 1998).

NA-COSs reacts with PAMP (pathogen-associated molecular pattern) and lipopolysaccharides (LPS) express the hypersensitive, genes expression and host-specific symbiosis between legumes in rhizobial plants (Cohn et al. 1998, Desaki et al. 2006). NA-COSs defense against pathogens, and elicitor in monocots and dicots (Shibuya and Minami 2001). Whereas, then modify NA-COSs reacts to legume roots in the symbiotic interaction with rhizobial bacteria (Truchet et al. 1991), which activate the phospholipase C (PLC), phospholipase D (PLD) and syntheses phytoalexin (PA), vertebrate somatic embryogenesis development (Bakkers et al. 1997). Whereas, *O*-acetylated NA-COSs are triggering cell division in the root cortex of *Vicia sativa* and delivered by ballistic micro targeting (Schlaman et al. 1997). Innate immune response, NA-COSs with PAMP (pathogen-associated molecular pattern) and with lipopolysaccharides (LPS) the hypersensitive gene's expression (Desaki et al. 2006).

Chitosan has been used in agriculture as a coating material for fruits, seeds, and vegetables (Lee et al. 2005, Photchanachai et al. 2006) for controlled agrochemical release of fertilizers (Sukwattanasinitt et al. 2001) to stimulate plant immune systems, plant growth, and plant production, and also to protect plants against attack by microorganism (Hadwiger et al. 2002). Chitosan is an exogenous elicitor of response mechanisms and has been demonstrated to induce plant defenses in tomato (Benhamou and Theriault 1992), cucumber (Ben-Shalom et al. 2003), chili seeds (Photchanachai et al. 2006), strawberry fruits (El Ghaouth et al. 1992), and rose shrubs (Wojdyla 2004). Several studies have shown that chitosan stimulates other systems involved in resistance of plants to infection (Kim et al. 2005).

40.3.2.3 Orchid Production

Orchid production is a leading demand for technical assistance in order to improve orchid growth, development, production, and quality. Chitosan is used in many industries, including waste water treatment, membrane technology, pulp paper, cosmetics, food industry, medical, biotechnology, and agriculture (Imeri and Knorr 1998). In agriculture, there is a worldwide trend to use chitosan as an alternative compound, because of its fungicidal effects and elicitation of defense mechanisms in many plant tissues. It is also used as a coating material for prolonging postharvest life and limits fungal decay on strawberry and bell pepper (Terry and Joyce 2004). Moreover, the use of the culture media supplemented with 1.75% (v/v) chitosan solution (chitogel) enhanced root, shoot biomass, photosynthesis, and related parameters of grapevine plantlets *in vitro* culture (Barka et al. 2004).

40.3.2.4 Miscellaneous Studies

Chitosan has reduced the transpiration in pepper plants, resulting in the reduction of water, while maintaining biomass production and yield. These results suggest that chitosan might be an effective antitranspirant to conserve water in agriculture (Bittelli et al. 2001). Increasing levels of abscisic acid (ABA), which plays a key role in the regulation of water use by plant resulted in closure of stomata and decreased transcription (Willmer and Fricker 1996). ABA and jasmonic acid have similar activity (Sembdner and Parthier 1993). Therefore, the stomata closure induced by foliar application of chitosan may influence pathways involving jasmonic acid or ABA. Azian et al. (2004) reported that vase solutions containing chitosan at 25 and 50 mg/L increased the vase life of cut *chrysanthemum* by 13 and 15 days, respectively, in comparison to control flowers that had a vase life of 6.8 days. The application of chitosan combined with ammonium carbonate offers a commercially acceptable, economically viable, and effective alternative for postharvest control of anthracnose in stored papaya. Dipping papaya in chitosan plus ammonium carbonate, significantly ($P < 0.005$) retarded color development of skin and flesh, increased fruit firmness, and reduced weight loss (Sivakumar et al. 2005). This chapter is intended to give an overview of the biosynthesis of constituent cell wall polysaccharides and their postsynthetic modification in *Aspergillus fumigatus*; it also discusses the antifungal drugs that affect cell wall polysaccharide biosynthesis.

40.4 CONCLUSIONS

In recent years, researchers are using chitin and chitosan derivatives for different purposes, among that for production of secondary metabolites under *in vitro* conditions as a renewable source for new drug development. In spite of the potential use of chitin and its derivatives, they have not been fully exploited for various plant biotechnology applications. Most of the studies have been done only at the horticultural aspects and a few *in vitro* studies have been envisaged. This chapter is intended to give an overview of valuable secondary metabolites production and large-scale plant production with the help of chitin and chitosan derivatives via *in vitro* and *in vivo* technology.

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41 Mechanism and Application of Chitin/Chitosan and Their Derivatives in Plant Protection

Heng Yin and Yuguang Du

CONTENTS

41.1 INTRODUCTION

Although chitin was isolated by Braconnot in 1811 for the first time from the cell walls of mushrooms, and chitosan was found in 1894 by Hoppe-Seyler, they remained unused biological resources for more than 100 years. However, interest in these abundant carbohydrates increased rapidly from the 1960s. The enormous increase in the number of relevant research papers and patents revealed a surprisingly high level of chitin/chitosan research activity from both academic and industrial scientists. Many literatures reported that CTS (chitin/chitosan and their derivatives) have antimicrobial and plant-defense elicit function (Albersheim and Darvill 1985); therefore, CTS are considered useful pesticides in the control of plant disease.

Based on these characteristics, CTS are used for protecting plants. Earlier, chemical pesticide was used in the traditional plant disease-insect-control ways; they got good results and problems simultaneously, especially the 3 "R" (resurgence, residue, resistance) problems. Not only people, but the environment too is affected by pesticides. So, many governments and people considered novel methods to develop pollution-free pesticides. CTS has great potential to be an effective green biopesticide. As a biopesticide, CTS can function as a seed-soaking agent, a root-applying agent, and a spray agent; all of these play an important role on plant disease control and stress resistance. However, chitin is not soluble in common solvents and chitosan is insoluble in either organic

solvents or water. So the soluble chitin/chitosan derivatives, especially their oligomers, have great application foreground on plant disease control. For example, oligochitosan has been granted pesticide license from the Ministry of Agriculture of China and is produced on a large scale. Today, oligochitosan has been used in more than 20 provinces in China, and about 143,000 ha is used for producing oligochitosan only at Liaoning, Hainan, and Shaanxi provinces (Zhao 2006).

This chapter aims to present an overview of the effect, mechanisms, and applications of CTS on plant protection. The recent growth in this field and the latest research papers published in this field will be introduced and discussed.

41.2 PLANT PROTECTION EFFECTS

The plant protection activity of CTS has been well documented in many different plant systems. The control diseases of CTS include bacteria, fungi, and viral diseases. In this section, the effect of CTS on several plants will be discussed.

41.2.1 Food Crops

In 1980, Professor Hadwiger at Washington State University reported that oligochitosan can induce soybean against *Fusarium solani* (Hadwiger and Beckman 1980). Oligochitosan from the *F. solani* f. sp. *phaseoli* cell walls could elicit defense reaction in pea pod tissue. Concentrations of oligochitosan as low as 0.9 and 3 mg/mL elicited phytoalexin induction and inhibited the germination of *F. solani*, respectively. When oligochitosan was applied to pea with or prior to *F. solani*, pea was protected from pathogen infection. This was the first publication of CTS-induced plant resistance. From then on, a series of excellent work was conducted in his laboratory. These creative researches led to an increased tide on the study of CTS-induced plant defense.

Rice is the staple food in the world, but many diseases such as rice blast pathogen cause enormous loss on both rice production quality and quantity. Great advancement of CTS on rice disease control has been achieved in recent years. In 2002, Agrawal and coworkers reported the effect of chitosan in initiating defense response in the leaves of rice for the first time (Agrawal et al. 2002). They found that after treatment with 0.1% chitosan, necrotic streaking was clearly observed on the upper side of rice leaves. These symptoms showed an increase in levels of the reactive oxygen species (ROS). Additionally, the production and accumulation of phenolic secondary metabolites was also upregulated after chitosan treatment. Enhanced defense against rice blast pathogen, *Magnaporthe grisea* 97-23-2*D*1, was observed in *H7S* rice seedlings treated with oligochitosan. In this experiment, 5 mg/L oligochitosan solution showed the best effect, the disease control effect was more than 50% (Lin et al. 2005).

Wheat is another important source of staple food, especially in cold countries; therefore, Russian scientists conduct more research in this area. Studies conducted on wheat infection with *Bipolaris sorokiniana* indicated that oligochitin with a molecular weight of 5–10 kDa and the degree of acetylation of 65% has good effect on controlling wheat disease (Khairullin et al. 2001, Burkhanova et al. 2007). In our laboratory, the ability of oligochitosan to promote wheat resistance to pathogenic toxin was validated (Liu et al. 2001).

41.2.2 Economic Crops

Tobacco is an important economic crop and a model plant for research. Many reports reveal that CTS can induce tobacco's resistance to tobacco mosaic virus (TMV), tobacco necrosis virus (TNV), and *Phytophthora parasitica nicotianae*. For example, Falcon studied the effect of different sizes and deacetylation degrees of chitosan derivatives on tobacco protection against *P. parasitica* (Falcon et al. 2008). The result of their experiment showed that different chitosans have distinct effects on this disease control: though less acetylated chitosan are better for inhibition of *P. parasitica*

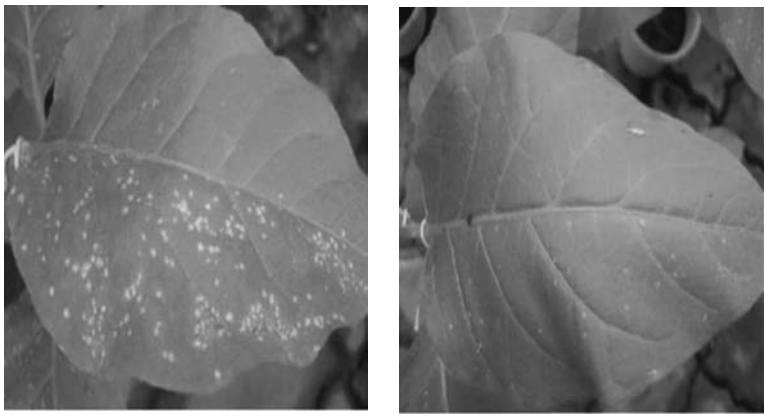

FIGURE 41.1 The effect of CTS on TMV control.

growth, partially acetylated chitosan are more effective in protecting tobacco against this pathogen by systemic induction of plant immunity. In another experiment, Iriti found chitosan application induced a high level of resistance against TNV; the chitosan treatment reduced the percentages of total lesion area per leaf by 95.2% in respect to control plants (Iriti and Faoro 2008). The effect of oligochitosan on TMV control was studied from the 1990s in our laboratory: oligochitosan was sprayed on tobacco leaves for the inhibition of TMV (Figure 41.1). The maximum inhibition of TMV was observed when inoculation occurred 24 h later after spraying 50 μg/mL oligochitosan solution (Zhao et al. 2007a).

Oilseed rape has a century-old planting history; it was cultivated in India 3000 years ago. Oilseed rape has a wide application in human food, animal feed, the chemical industry, and regenerative energy. As a suitable bioenergy, the importance of oilseed rape has thus increased in recent years. *Sclerotinia* rot is the most harmful disease on oilseed rape production. The inducing resistance of oligochitosan to *Sclerotinia sclerotiorum* on *Brassica napus* was studied in our laboratory. Whereas oligochitosan did not affect the radial growth of *S. sclerotiorum* colonies on PDA plates, it reduced the frequency and size of rot compared to untreated controls when applied to oilseed rape before inoculation with *S. sclerotiorum*. The best pretreated time was 3 days before inoculation with *S. sclerotiorum*, and the best inducing resistance concentration of oligochitosan was 50 μg/mL (Yin et al. 2008a). Oligochitosan can be modulated into steady colloid solution, so it can be used as a seed-coating agent. It does not influence the seed sprout and emerge, but can obviously suppress the emergence of *S. sclerotiorum*; the control rate of three species of rape was 34.19%–44.10% (Lu et al. 2003).

Potato is a tuberous crop from the perennial *Solanum tuberosum* of the *Solanaceae* family. It is an essential crop in Europe and Asia. China is now the world's largest potato-producing country. The effect of the chitosan-induced resistance to viral infection was investigated in potato plants. The plants were sprayed with different molecular weights of chitosan solution (1 mg/mL) and the greatest antiviral activity was shown by 120 kDa chitosan. In another experiment, potatoes were infected with potato virus X after chitosan pretreatment. It was found that chitosan treatment significantly decreased the number of systemically infected plants compared to control, and the treated leaves also accumulated less amount of virus than the control leaves (Chirkov et al. 2001).

41.2.3 Vegetable Crops

The effect of oligochitosan and oligochitin on gray mould caused by *Botrytis cinerea* in cucumber plants were evaluated by Ben-Shalom. It was shown that oligochitosan and oligochitin had different effects on this cucumber–pathogen interaction. Although complete inhibition of *Botrytis*

conidia germination was found at 50 ppm chitosan solution in vitro, chitosan also controlled the gray mould caused by *B. cinerea* in treated plants compared with control plants. But there was no effect of oligochitin on both pathogen growth on PDA and leaves. Besides this fungicidal effect, spraying chitosan 1, 4, and 24 h before inoculation with *B. conidia* decreased gray mould by 65%, 82%, and 87%, respectively. However, spraying chitosan on the leaves 1 h after inoculation decreased gray mould incidence only by 52%. These results suggested that the antifungal and elicitor activity of CTS are both necessarily for the control of gray mould in cucumber (Ben-Shalom et al. 2003). *Pythium aphanidermatum (Edson) Fitzp* is another plant pathogen that causes root and crown rot in cucumber. A recent paper showed that chitosan cannot induce defense to this pathogen when used alone. But the application of chitosan in combination with *Lysobacter enzymogenes* 3.1*T*8 (a biocontrol bacterium) reduced the number of diseased plants by 50%–100% (Postma et al. 2009). It is concluded that the addition of chitosan can enhance the biocontrol efficacy of biocontrol bacteria.

Vasyukova reported that low molecular weight chitosan displayed an elicitor activity by inducing the local and systemic resistance of *Lycopesicon esculentum* tomato to *Phytophthora infestans* and nematodes (Vasyukova et al. 2001). Other studies indicated that chitosan has the ability to protect tomato plants against *Colletotrichum* sp. Chitosan significantly inhibited the radial growth of this fungus with a marked effect when the concentration is higher than 1.5%. When tomato fruits treated with 1.0% and 2.5% (w/v) chitosan were artificially inoculated with *Colletotrichum* sp., after 10 days at 24°C, chitosan significantly reduced the lesion size of tomato fruits (Munoz et al. 2009).

41.2.4 Fruits

Grape is a familiar nourishing fruit; it can be eaten raw or used for making jam, juice, wine, grape seed extracts, raisins, and grape seed oil. Grape production is severely damaged by many pathogens such as *B. cinerea*. CTS are used as biopesticide in many grape-producing countries. Aziz in the University of Reims reported that oligochitosan at 200 μg/mL with a molecular weight of 1500 and a degree of acetylation of 20% dramatically reduced the infection of grapevine leaves by *Plasmopara viticola* and *B. cinerea*. Dose–response experiments showed that maximum defense reactions and control effect of *B. cinerea* were achieved with 75–150 μg/mL oligochitosan. However, greater concentrations of oligochitosan did not present the same efficiency but the oligochitosan and bluestone cotreatment has high efficiency (Aziz et al. 2004, Trotel-Aziz et al. 2006). Similarly, on apple and watermelon, CTS can induce plant defense against canker and anthracnose. In an experiment conducted in our lab, after chitosan treatment, the infected areas of apple trees were cured (Figure 41.2).

FIGURE 41.2 The effect of CTS on apple canker control.

41.2.5 Summary

It has also been reported that CTS can activate plant defense to disease on several other plants such as barley (Faoro et al. 2008), pearl millet (Manjunatha et al. 2008), carrot (Chris et al. 2004), sunflower (Nandeeshkumar et al. 2008), coconut (Lizama-Uc et al. 2007), etc. In general, there are five characteristics of CTS mediate plant defense:

1. CTS are effective for the treatment of plant–disease interaction in many cases; the experiments in our lab found that CTS can produce resistance to more than 40 diseases on 24 plants.
2. Different members of CTS have different effect on plant protection. Aiming at different diseases, the most effective concentration, polymerization degree, and deacetylation degree of CTS are different. This phenomenon originates from the distinct action mechanism of different plant–pathogen interaction.
3. Both the antimicrobial activity and elicitor activity of CTS are necessarily for the disease control.
4. Pretreatment of the plants with CTS before pathogen infection will result in better plant protection than CTS treatment after pathogen infection. This is accord with the PRIME mechanism in induced systemic resistance of plants. PRIME mechanism has been put forward during the past several years (Conrath et al. 2006); it has been demonstrated that pretreatment of plants with elicitor of systemic resistance leads to the direct activation of a set of defense reactions and also primes the plants for stronger elicitation when it is affected by disease at a later stage.
5. CTS can be used alongside other pesticides or biocontrol agents.

41.3 MECHANISMS OF CTS IN PLANT PROTECTION

Though many application experiments have been carried out, the mechanism how CTS induces the resistance of plants to pathogens is still unclear. Studying the mechanism will provide evidences for application of CTS on crop protection. Scientists have been trying to explore the mechanism of CTS-induced plant-defense responses from the 1990s. Numerous works indicate that the plant protection function of CTS comes from the antimicrobial activity and plant innate immunity elicited activity of CTS. The antimicrobial activity was discussed in some excellent review papers (Cuero 1999, Rabea et al. 2003), so this paper focused on the mechanism how CTS induced plant immunity. Based on our experiments and other literature, we conclude that there are five steps in the process of CTS-activated plant innate immunity. This aspect, especially the CTS signal reception and transduction, will be described in detail in the following.

41.3.1 Signal Reception

Signal reception is the first challenge for the plant innate immune system startup. Perception of elicitors by an array of pattern recognition receptors comprises the basis for communication between plants and elicitors in a number of studied plant systems. Researchers have found that different members of CTS have different receptors.

The binding proteins of chitin or oligochitin were found on several plants such as tomato, rice, and soybean. In 1991, Baureithel first reported that there are high-affinity binding sites of penta-*N*-acetylchitopentaose at the surface of tomato cells as well as in microsomal membranes prepared from these cells (Baureithel et al. 1994). The same phenomenon was observed by Shibuya in the plasma membrane from suspension-cultured rice cells (Shibuya et al. 1996). Both these results suggested that there is a receptor of chitin or oligochitin on plant membrane. From 1997 to 2001, high-affinity binding proteins of oligochitin were identified from rice, soybean, wheat, barley, and

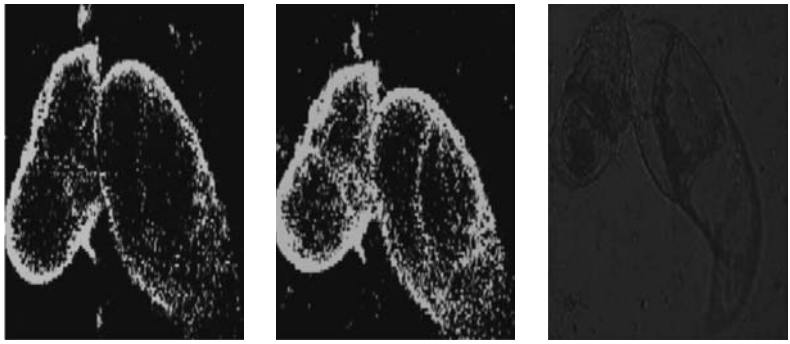

FIGURE 41.3 The binding process of labeled oligochitosan to strawberry cells. (A) The binding of 2-AMAC-oligochitosan to strawberry cells in 2 min; (B) the binding of 2-AMAC-oligochitosan to strawberry cells in 10 min; (C) strawberry cells screened with visible light.

carrot by photoaffinity labeling and affinity cross-linking of carbohydrate and protein techniques in Shibuya's lab (Ito et al. 1997, Day et al. 2001, Okada et al. 2002).

Further results were obtained in recent years. Kaku et al. (2006) reported the purification of an oligchitin binding protein (CEBiP) from plasma membrane of rice cells and the full-length cloning of its gene. CEBiP is a glycoprotein consisting of 328 amino acid residues and glycan chains, the total molecular mass is 75 kDa. CEBiP has specific binding with oligochitin but not with oligochitosan. In CEBiP-RNAi lines, where expression of this gene was knocked down, the defense-related gene responses as well as elicitor-induced oxidative burst was suppressed, showing that CEBiP plays a key role in the perception of oligochitin in rice cells. Structural analysis indicated CEBiP does not contain any intracellular domains suggesting that another component is required for signal transduction through the rice plasma membrane into the cytoplasm. In recent papers, two research groups found a receptor-like kinase, designated CERK1 or LysM RLK1, which is essential for oligochitin elicitor signaling in *Arabidopsis* (Miya et al. 2007, Wan et al. 2008). This kinase is a plasma membrane protein containing an intracellular Ser/Thr kinase domain and three LysM motifs in the extracellular domain. These results suggest that a receptor-like kinase and CEBiP may cooperate, which plays a critical role in oligochitin perception.

Interestingly, there are few research papers on the binding protein or receptor of chitosan or its oligomer. A lectin-specific protein for oligochitosan was purified by chitosan affinity chromatography from cultured cells of *Rubus* by Lienart in 1991 (Liénart et al. 1991). But the presence of binding protein for chitosan or oligochitosan on other plants is still unclear. In our lab, we found there is high-affinity binding of oligochitosan to strawberry, tobacco, and oilseed rape cells by using fluorescent-labeled oligochitosan; time response was also observed in this experiment (Figure 41.3), the binding protein identification is in process.

41.3.2 Signal Transduction

After CTS signals enter the cytoplasm via receptors, the CTS signals are transmitted quickly and build up a complicated network. Much work has been done to elucidate this signal network, but it is still unclear. According to previously published papers, Ca^{2+}, ROS, nitric oxide (NO), abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) all take part in CTS signal pathway.

Klüsener reported that chitosan can induce elevations in the concentration of free cytosolic calcium in guard cells of *Arabidopsis* (Klüsener et al. 2002). In other studies carried out with cotton, Guo found that treating tobacco with oligochitosan (DP = 3–7) and $LaCI_3$ (a Ca^{2+} channel blocker) or trifluoroacetic acid (a calmodulin antagonist) will depress the upregulated effect of oligochitosan on

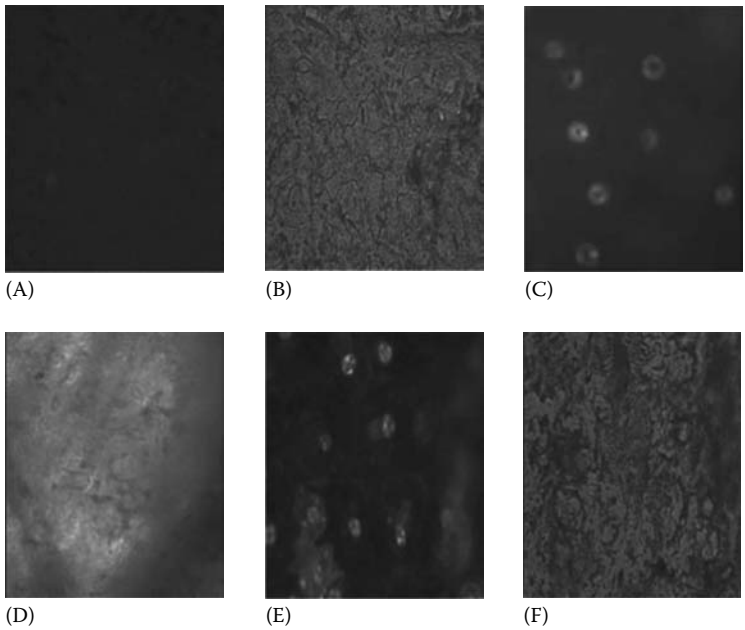

FIGURE 41.4 CTS-induced production of H_2O_2 and NO in epidermal cells of *B. napus* leaves. A, C, E: Fluorescent images; B, D, F: Bright field images; (A, B) Control; (C, D) Oligochitosan treated and H2DCF-DA labeled; (E, F) Oligochitosan treated and DAF-2DA labeled.

defense-related enzyme activity (Guo et al. 2004). All these results revealed that Ca^{2+} is an important factor in CTS signal transduction.

ROS and NO are also considered as important defense signal molecules (Zaninotto et al. 2006). Among different ROS, hydrogen peroxide (H_2O_2) is the most important one; it can cross membranes and can directly function in cell signaling. In our lab, oilseed rape was used to investigate oligochitosan inducing production of H_2O_2 and NO (Figure 41.4) by using fluorescent probe H2DCF-DA and DAF-2DA. The result showed that the production of H_2O_2 and NO in epidermal cells of oilseed rape were induced with oligochitosan in short time. And it was proved that there was interaction between H_2O_2 and NO by using *L*-NAME (an inhibitor of NOS that also inhibits plant NO synthesis) and catalase (an H_2O_2 scavenger), respectively (Li et al. 2009). Similar result was obtained in experiments with tobacco in our lab (Zhao et al. 2007b).

Dose response was observed during oligochitosan-induced production of H_2O_2 in many plant systems. For example, the production of H_2O_2 could be detected when the concentration of oligochitosan was 15 mg/mL, reached a maximum at 60 mg/mL, and decreased at higher concentrations when oligochitosan of molecular weight 1335 Da was used to treat rice cells. A time response was also observed in this experiment, the generation of H_2O_2 reached a maximum near 1 h, then decreased over the next 2 h (Lin et al. 2005). Srivastava found that chitosan can induce ROS and NO production in abaxial epidermis of *Pisum sativum*. The calcium chelators, BAPTA-AM or BAPTA, did not have a significant effect on the chitosan-induced rise in ROS or NO revealed Ca^{2+} isn't participate in this ROS or NO production process in *P. sativum* (Srivastava et al. 2009).

JA and SA are plant hormones required for signal transduction leading to plant resistance to pathogens and insects (Koornneef and Pieterse 2008), JA mediate induced systemic resistance (ISR), and SA mediate systemic acquired resistance (SAR). Previous studies found that CTS can elicit these hormones, especially JA production on plant. Doares reported JA levels in leaves of tomato plants increased several fold within 2 h after supplying the oligochitosan (DP = 4) to the plants through their cut stems (Doares et al. 1995). Similar results were obtained with rice: transient OPDA (12-oxo-phytodieonic acid, precursor of JA) and JA accumulation was detected within 3 min

in chitosan-treated rice leaves. OPDA peaked quickly around 5 min and returned to its basal level within 15 min, whereas JA induction upon chitosan treatment peaked 60 min and decreased within 360 min (Rakwal et al. 2002). The concentrations of several plant hormones in oligochitosan-treated tobacco leaves were determined by high-performance liquid chromatography with a coulometric array detector in our lab. The hormone concentrations changed with the treating time by oligochitosan: JA and gibberellic acid reached peak value after being treated for 6 h and indoleacetic acid after 8 h (Du et al. 2003). It is concluded that CTS induced plant innate immunity through the JA/ET pathway; however, other reports mentioned a contrary effect. For example, Obara found MeSA was accumulation in rice leaves after chitosan treatment for 7 h (Obara et al. 2002). MeSA is biosynthesized from SA, which plays an important role in SA-mediated SAR.

ABA is another important plant hormone that participates in plant diseases and stress resistance (Mauch-Mani and Mauch 2005). The role of ABA in CTS-induced plant immunity was studied by Iriti and Faoro (2008). Chitosan application induced ABA accumulation in leaf tissues at 24 h after treatment, and elicited resistance against TNV. Besides, treatment with nordihydroguaiaretic acid (an ABA inhibitor) before chitosan application reduced tobacco resistance to the necrosis virus. It is indicated that ABA plays an important role in CTS-induced resistance mechanism.

In general, these results suggest that the CTS defense signal network is mediated by several signaling pathways. The reason of this phenomenon maybe due to the variety of plant–disease interactions, so the plants have induced resistance to different diseases through different pathways. It is also proved that CTS are broad-spectrum, plant-defense elicitors.

41.3.3 CTS Response Genes

The CTS response genes is another research hotspot of CTS-induced plant defense. In our lab, mRNA differential display was used to investigate oligochitosan-induced transcriptional activation of defense-related genes. Accordingly, a novel Ser/Thr protein kinase gene was isolated and designated as oligochitosan-induced protein kinase (*oipk*) (Feng et al. 2006). A plant *oipk* antisense expression vector was constructed and transformed into tobacco by *Agrobacterium tumefaciens*. Decreased phenylalanine ammonia-lyase activity and decreased resistance to TMV were observed in this transgenic tobacco. These results indicate that *oipk* is involved in the signal pathway of oligochitosan-induced resistance in tobacco. Furthermore, many important plant defense or signal-related genes such *MAPK, SKP*1 were found upregulated by CTS in rice, tobacco, oilseed rape, etc. (Zhang et al. 2007, Lee et al. 2008, Yin et al. 2008b).

Since its appearance in 1996, DNA microarray has been widely used for monitoring gene expression. It was also used for CTS response gene detection. Ramonell investigated gene expression patterns in *Arabidopsis* in response to chitin treatment using an *Arabidopsis* microarray consisting of 2375 expressed sequence tag (EST) clones representing putative defense-related and regulatory genes (Ramonell et al. 2002). Transcript levels for 61 genes were changed threefold or more in treated plants relative to control plants. In these 61 genes, some are involved in cell-wall strengthening and cell-wall deposition. Expression of genes expression alters caused by *N*-acetylchitooctaose was analyzed by using a microarray containing 8987 randomly selected rice ESTs (Akimoto-Tomiyama et al. 2003). In this experiment, 166 genes were significantly induced and 93 genes were repressed. Of the 259 genes identified as responsive to *N*-acetylchitooctaose, 18 genes are related to signal transduction, including 5 important signal pathway element calcium-dependent protein kinases. An oilseed rape cDNA microarray containing 8095 ESTs was used to analyze the *B. napus* genes expression alters elicited by oligochitosan (Figure 41.5). Transcriptal levels for 393 genes were altered twofold or more in oligochitosan-treated plants compared to control plants. Of the 393 genes, 136 were induced and 257 were repressed. These 393 genes were involved in different processes and had different functions including primary metabolism, transcription, defense, and signal transduction. An important JA synthase (2-oxophytodienoate-10,11-reductase) gene, some genes which regulated the JA/ET pathway (like *MPK*4, *EREBP*), and several JA-mediated genes

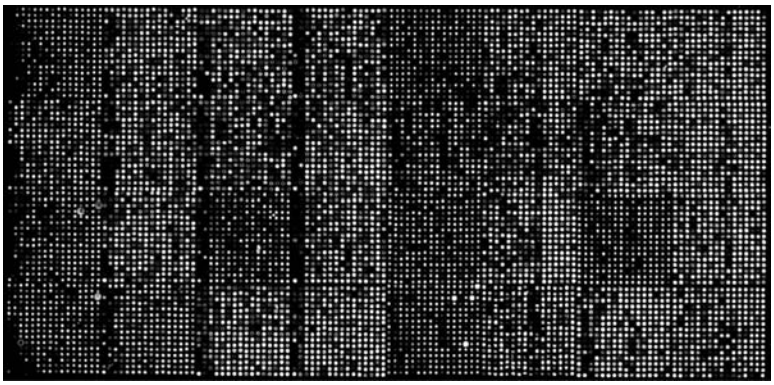

FIGURE 41.5 The *B. napus* cDNA microarray was hybridized with fluorescence-labeled mRNA preparation from oligochitosan treated and control plants.

were induced by oligochitosan, suggesting that oligochitosan activated the plant innate immunity in oilseed rape through JA/ET signaling pathway (Yin et al. 2006).

41.3.4 CTS Response Proteins

Lots of literatures showed that CTS can induce some defense-related proteins and enzyme activity such as chitinase, glucanase, and phenylalanine ammonia-lyase. For example, a bioassay in rice seedlings to test plant protection demonstrated that chitosan and oligochitosan can induce higher levels of glucanase activity than control seedlings (Rodriguez et al. 2007). Similar results were obtained with tobacco by Falcon et al. (2008). Bohland et al. (1997) reported that both oligochitin and chitosan can induce the activity of lipoxygenase (an enzyme participate in JA synthesize) in wheat cells. In our lab, we also found the activities of catalase, phenylalanine ammonia-lyase, peroxidase, polyphenoloxidase, and superoxide diamutase in *B. napus* leaves were upregulated after oligochitosan treatment (Yin et al. 2008c).

Nowadays, proteomics has become one of the most powerful tools for identifying responded protein in plants. In order to get more information about plasma membrane proteins involved in chitosan-induced rice defense responses, plasma membrane proteomic analysis of the rice cells elicited by chitosan was conducted. A total of 14 up- or down-regulated protein spots were observed at 12 or 24 h after chitosan treatment. Most of these proteins are related to signal transduction (Chen et al. 2007). Ferri reported that chitosan treatment strongly increased the expression of 11 proteins of the pathogenesis-related protein-10 family by using two-dimensional gel electrophoresis (Ferri et al. 2009).

41.3.5 Secondary Metabolites Production

The effect of CTS on plant defense–related secondary metabolites accumulation was studied from the 1980s. In 1980, Hadwiger and Beckman (1980) found chitosan from *F. solani* cell-wall fractions can induce a phytoalexin (pisatin) in soybean pod in 24 h. Similar results were obtained in parsley (Conrath et al. 1989) and *Catharantus roseus* (Kauss et al. 1989). We also investigated that oligochitosan can induce the accumulation of chlorogenic acid and rutin in tobacco leaves (Wang et al. 2007). In a recently published paper, the elicitation of coumarins and alkaloids accumulated by *Ruta graveolens* shoots after treatment of chitin and chitosan was studied in detail (Orlita et al. 2008). Both these two compounds induced a significant increase in the concentrations of nearly all the secondary metabolites. Adding 0.01% chitin caused the increase of several members of coumarin such as pinnarin, rutacultin, bergapten, isopimpinellin, and psoralen. Adding 0.1% chitosan induced production of not only simple coumarins but also furanocoumarins and dihydrofuranocoumarins.

Besides these phytoalexins, CTS can also lead to the production of lignin and callose. The accumulation of lignin and callose will reinforce the plant cell wall and enhance the structural resistance of plant. In suspension-cultured cells of *Glycine max*, the synthesis of callose started within 20 min of treatment with chitosan and persisted over hours (Köhle et al. 1985). Recent works indicated that the callose apposition caused by chitosan is related to ABA accumulation (Iriti and Faoro 2008). Vander studied the ability of oligochitin, oligochitosan (different molecular weight and deacetylation degree) to elicit lignin deposition in wheat leaves (Vander et al. 1998). They found that all oligochitosan but not the oligochitin induced the deposition of lignin, which suggested that oligochitosan has better structural resistance–activated function in wheat. In another study in our lab, we also found that oligochitosan can induce the lignin production in the root and leaves of cotton seedlings (Zhao 2006).

41.3.6 Summary

Though much work has been done to elucidate the mechanism of CTS-induced plant defense, there is still some uncertainty regarding the mechanism. From existing publications, we only know some elements and steps of this signal network, but cannot draw an integrated map of it. Many questions such as the identification of chitosan receptor still remain unanswered, and further research on the mechanisms of CTS-induced defense is needed.

41.4 CONCLUSION AND PROSPECT

CTS is widely used in plant protection from the 1990s. Researchers found that CTS can elicit defense to more than 60 diseases on several plants. The potent effect of CTS on plant diseases control is from its antimicrobial and plant innate immunity elicited activity. Though there are many papers focused on CTS and plant protection, there are still many problems that need to be studied. The vital problems to be solved are

1. The best concentrations and treat time of CTS in different diseases and plants.
2. The cooperated effect of CTS and other pesticides on plant diseases control.
3. The signal perception of CTS, especially the receptors validation.
4. The signal network of CTS-induced plant defense.

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42 Enhancing Crop Production with Chitosan and Its Derivatives

Nguyen Anh Dzung

CONTENTS

42.1 BIOACTIVITIES OF CHITIN, CHITOSAN, AND THEIR DERIVATIVES ON PLANTS

42.1.1 Chitin, Chitosan, and Their Derivatives as Novel Plant Regulators

For a long time, it was thought carbohydrates played only important roles as energy or structural components of plant cell wall. Recently, oligosaccharins have been known as biological signals for cells. Oligosaccharides, including chito-oligosaccharides, are cell molecular signals, which induce and regulate defensive, symbiotic, and developmental processes in plants (Darvill 1992, Cote and Hahn 1994, John et al. 1997).

Lipo-chitooligosaccharides (LCOs) consist of a tetra or pentameric chitooligosaccharide backbone, which is attached with a long chain unsaturated fatty acid, and play a key role in the first step of symbiotic nitrogen fixation and induce NOD gene that regulates the formation of nodules in leguminous plants at a very low concentration (Lerouge 1990). The biological activities of LCOs are not only limited to leguminous plants but also serve as plant-growth regulators. LCOs promote cell division in protoplasts grown in the absence of auxin and cytokinin and activate the expression of AX 11, a gene related to auxin signal (John et al. 1997).

It is known that there are five plant-growth regulators such as auxin, gibberellin, cytokinin, ethylene, and abscisic and, recently, oligosaccharins have been found to be novel plant-growth regulators. Oligosaccharins such as oligogalacturonic, chito-oligosaccharides, and *N*-acetyl oligoglucosamine are molecular signals that induce and regulate some genes of plants. Chitosan and chitosan oligomer are molecular signals that serve as plant-growth promoters and develop disease-resistance systems in plants. Chitosan oligomer can activate more than 20 pathogenesis-related

genes as defensins, ARnases, chitinases, β-glucanase, phytoalexins, lignins, and some gene-related metabolisms of plants (Hadwiger et al. 2002).

Due to the double effects on plants as plant-growth regulators and induction of plant defensive systems, chitosan and chitosan oligomer were used for plant tissue cultivation by supplement into cultivation media. The experiment was done with the addition of 10, 50, and 90 mg of chitosan glutamate per liter of the cultivation medium used for somatic embryo–derived plantlet of carrot (*Daucus carota* L); it was shown that the addition of 100 mg of chitosan glutamate per liter of the medium led to a relatively high somatic embryo–derived plantlet conversion 4 weeks after fluid drilling, and this conversion was also sustained until 7 weeks (Kitto et al. 1991).

Radiation-degraded chitosan was supplemented into plant tissue cultivation medium as a plant-growth promoter. Chitosan with a degree of deacetylation of 80% and M_w of 150 kDa was irradiated with gamma rays of 50, 100, 150, and 200 kGy and supplemented with a concentration of 50 ppm into the medium of plant tissue cultivation. There were five plant species, carrot, strawberry, *Limonium latifolium*, *Eustoma grandiflorum*, and *Chrysanthemum moriflorum*, that were used for studying the biological effects. The results showed that chitosan irradiated with 100 kGy introduced the strongest growth-promoting effect for plants in vitro. Fresh biomass of callus of carrot was shown to increase by 37%; fresh biomass of root also increased by 32% in the case of carrot, 26% in the case of strawberry, and 32.3% in the case of *L. latifolium*; in shoot height parameter, carrot increased by 25%, strawberry by 23%, and *L. latifolium* by 9.2%. The result also indicated that the suitable concentration of irradiated chitosan with 100 kGy was 50 ppm for *C. moriflorum*, 30–40 ppm for *L. latifolium*, and 50–100 ppm for *E. grandiflorum* (Luan 2002). Chitosan with M_w of 48 kDa and DD 80% was also irradiated by gamma rays at dosage up to 200 kGy and M_w of chitosan reduced from 48 to 9.1 kDa. It was found that chitosan irradiated at 100 kGy with an M_w of 16 kDa showed the strongest effect on the growth of plants in vitro. The optimal concentration of irradiated chitosan for the growth of shoot clusters and shoot multiplication rate was 70 mg/L for *Chrysanthemum*, 50–100 mg/L for *Lisianthus*, and 30–100 mg/L for *Limonium*. Fresh biomass increased by 68.1% for *Chrysanthemum*, 48.5% for *Lisianthus*, and 53.6% for *Limonium* (Luan et al. 2005). A comparison of the above results showed that the molecular weight of irradiated chitosan is smaller, and the bioactivity of plant-growth promotion is stronger.

Radiation-degraded chitosan with molecular weight of 45 kDa, DD > 80% was found to act as a plant-growth stimulator in orchid tissue cultivation. It was found that the optimal concentration of the chitosan oligomer of 50–75 ppm was the most effective for plant-growth promotion (Upathum 2002).

Chitosan was used as a growth stimulator for tissue culture of orchid *Dendrobium phalaenopsis*. Nge et al. 2006 studied effect of shrimp and fungal chitosan on the growth and development of orchid plants in tissue culture. Chitosan was used with molecular weight of 1, 10 and 100 kDa and chitosan concentration of 5, 10, 15, 20 and 25 ppm supplemented in the culture medium. The result showed that the shrimp chitosan oligomer of 1 kDa was more effective than the shrimp of 10 and 100 kDa in fresh weight of protocorm-like bodies of the orchid, and the optimal concentration of shrimp chitosan of 1 kDa was 15 ppm. Comparing the effectiveness of plant-growth stimulation of chitosan from shrimp and fungi, fungal chitosan of 10 kDa was the most effective in enhancing the fresh weight of protocorm and the number of plantlets generated by orchid protocorm (Nge et al. 2006).

Chitosan oligomer was also used as an elicitor to stimulate the accumulation of secondary metabolites as taxol from suspension cultures of *Taxus cuspidate* (Li and Tao 2009), anthraquinones in *Rubia tinctorum* L. (Vasconsuelo et al. 2004). Chitosan from crab shell with DD > 85% was supplemented into cell culture medium of *R. tinctorum* at a final concentration of 200 mg/L. Chitosan as an elicitor increased in anthraquinone production up to 110% compared to the control.

42.1.2 Chitin, Chitosan, and Their Derivatives Enhance Content of Chlorophyll

Chitosan oligomer was used widely as foliar for some plants such as peanut, soybean, coffee, black pepper, pepper, pearl millet, and cabbage (Bittelli et al. 2001, Wongroung et al. 2002, Dzung 2003, 2004a, 2004b, 2005, 2006, 2007, Sarathchandra and Jaj 2004, Uddin et al. 2004, Limpanavech et al. 2008).

In plants, photosynthesis occurs primarily in leaf cells in organelles called chloroplasts, which contain chlorophyll a, b and carotenoids. Chlorophyll and carotenoids play important roles in photosynthesis to produce sugars and organic molecules by fixation of CO_2 and water. It has been found that spraying chitosan and chitosan oligomer on the leaf of plants enhanced the content of chlorophyll and intensity of photosynthesis. Chitosan oligomer with molecular weight of 2 kDa was sprayed on peanut and soybean, and the results obtained showed that chitosan oligomer increased the chlorophyll content of soybean and peanut by 17.9% and 23.0%, respectively. The concentration of chitosan oligomer suitable for increasing the content of chlorophyll of soybean and peanut was 30 ppm, the same optimal concentration for plant-growth promotion (Dzung 2004a,b, 2005). For industrial and fruit plants, such as coffee and black pepper, optimal concentration of chitosan oligomer (2 kDa) was 40–50 ppm (Dzung 2007).

Effects of chitosan and chitosan oligomer on increasing the chlorophyll content of plants are shown in Table 42.1.

Nitar et al. (2004) studied soaking rice seed in chitosan oligosaccharide at concentration of 20 ppm for 1 day. She observed that the treated leaves of rice was greener than the control and suggested that chitosan oligosaccharide may enhance the synthesis of chlorophyll in plant.

Effect of chitosan oligomer with molecular weight of 2 kDa on content of chlorophyll and carotenoid of coffee seedlings in greenhouse was shown in Table 42.2. It was found that the effect of chitosan oligomer on the content of chlorophyll and carotenoid of coffee increased the content of chlorophyll total up to 40%–65% at 40–60 ppm (Toan 2008 unpublished).

Limpanavech et al. (2008) also used chitosan with M_w of 45 kDa, 90%DD at 1, 10, 50, and 100 ppm to spray on *Dendrobium* orchid plants. It was found that the chitosan increased significantly

TABLE 42.1
Effect of Concentration of Chitosan Oligomer on Contents of Chlorophyll in the Leaves of Some Plants

Conc. of Chitooligomer	0 ppm	20 ppm	30 ppm	40 ppm	50 ppm	80 ppm	100 ppm	References
Peanut	4.35	5.42	5.75	5.18	5.08	—	—	Dzung (2004a,b)
Soybean	2.76	3.16	3.23	3.10	2.92	—	—	Dzung (2005)
Coffee[a]	2.24	2.27	2.31	2.59	2.14	—	—	Toan (2007)
Coffee[b]	2.21	2.24	2.13	2.18	2.453	2.52	—	Dzung (2007)
Coffee seedling	1.05	1.15	—	1.43	1.70	1.38	—	Nam (2008)

[a] Coffee: with chitosan oligomer.
[b] Coffee: with chitosan.

TABLE 42.2
Effect of Concentration of Chitosan Oligomer on Contents of Chlorophyll and Carotenoid of the Leaves of Coffee Seedlings in Green House (Unit: mg/1 g of Fresh Leaf)

Conc. of Chitosan Oligomer	0 ppm	20 ppm	40 ppm	60 ppm	80 ppm
Chlorophyll a	0.70	0.77	0.95	1.18	0.91
Chlorophyll b	0.35	0.38	0.48	0.56	0.47
Total chlorophyll	1.05	1.15	1.43	1.74	1.38
Carotenoids	0.44	0.51	0.66	0.76	0.63

the chloroplast diameter of *Dendrobium* orchid at concentration of 10–50 ppm in young leaves and at 50 ppm in old leaves. Diameter of chloroplast was of 9.15 μm (control) and increased up to 12.16 μm at 10 ppm in young leaves and from 7.97 μm (control) up to 10.43 μm at 50 ppm in old leaves. Limpanavech also found that chitosan effected on chloroplast gene expression, therefore chitosan increased in chloroplast size and enlargement of chloroplast. This might be one of the factors led to stimulating the growth of plants.

Chibu and Shibayama (2001) applied chitosan at concentration of 0.1%–0.5% in soil-planted soybean, upland rice, mini-tomato, and lettuce. The results showed that there was increase in chlorophyll density of chitosan-treated plant compared to the control. The leaves of the plant treated with chitosan were more dark green than the control.

E. grandiflorum was treated with chitosan and sugar as fructose, glucose, and galactose. It was found that chitosan promoted the accumulation of anthocyanin in petals in vitro and increased the quality of the flowers (Uddin et al. 2004).

42.1.3 Chitin, Chitosan, and Their Derivatives Increase in Number of Nitrogen Fixing Nods of Leguminous Plants

As discussed in Section 42.1.1, LCOs play a key role in the first step of symbiotic nitrogen fixation. They are molecular signals which induce NOD gene that regulates formation of nodules in *leguminous* plants at very low concentrations (Lerouge 1990). This bioactivity of chitosan oligomers has also been demonstrated in experiments.

Chitosan with molecular weight of 100 kDa at concentration of 0.5% was added to the soil and the number of nodules of soybean increased by 5.0/plant compared to 4.25/plant in the control.

Chitosan and chitosan oligomer solution coated soybean seeds for 30 min before plantation (Dzung 2002). Effect of chitosan and chitosan oligomer (1–2 kDa) on the number of nitrogen fixing nodules is shown in Table 42.3.

The results shown in Table 42.3 indicated that chitosan and chitosan oligomer effected clearly on the number of nodules of soybean, but chitosan oligomer stimulated strongly than the chitosan. Additionally, chitosan and chitosan oligomer increased the effective rate of nodules.

Chitosan oligomer solution in acetic acid 0.5% was also sprayed on the leaves of peanut at concentrations of 20, 30, 40, and 50 ppm with three times of application per crop. Similar to the results of soybean application, the results on peanut also showed that chitosan oligomer (1–2 kDa) increased in the number of nodules of peanut, and increased by 50% at concentration of 40–50 ppm compared to the control (Dzung 2004a,b).

42.1.4 Stimulating Seed Germination

One of the most important bioactivity of chitin, chitosan, and their derivatives on plants is stimulation of seed germination. Since 1996, this bioactivity has been applied in agriculture (Hadwiger 1986, Yano 1988). Derivatives of chitin as depolymerized chitin, CM-chitin, and hydroxyethyl-chitin were used to coat soybean seed and then cultured in the field. It was observed that the rate of germination of soybean seed increased by 6.0% compared to the control (Hirano 1996). A detailed study of DD%

TABLE 42.3
Effect of Chitosan and Chitosan Oligomer on the Number of Nitrogen Fixing Nodules of Soybean

Experiments	Water	Chitosan	Chitosan Oligomer
Nodule total/five plants	224.6	318.3	457.3
Effective nodules/five plants	154.6	254.6	420.3
Effective rate, %	68.8	79.9	91.9

and concentration of chitosan and organic acid on seed germination rate of alfalfa, lettuce, mung bean, green pea, radish, and spoon cabbage was carried out by Li and Wu (1998). The seed of plants were immersed in chitosan solution at concentration from 0.5% to 1.5% for 30 s. The results obtained showed a different effect for each plant. Chitosan solution did not effect on stimulating the seed germination rate of alfalfa, wheat berry, green pea, mung bean, and radish. Chitosan with 80% DD at concentration of 1.0% in lactic acid was found to be more effective to Chinese kale, spoon cabbage, amaranthus, and cauliflower, and 1.5% chitosan with 90% DD in lactic acid was also effective to lettuce and celery.

Wheat seed was also coated by chitosan solution at concentration from 2 to 8 mg/L for 15 min. It was found that germination rate of untreated seeds was 50%–57% and chitosan-treated seed was 70%–90%. The difference of germination rate increased significantly from 2 to 8 mg/L compared to the control (Reddy et al. 1999). The optimal concentration of chitosan was found to be 15 ppm for increase in the germination rate of cucumber, chili, pumpkin, and cabbage (Chandrkrachang 2002).

Chitosan solution 4% with trade name Elexa (from Boston, Massachusetts) was used to treat pearl millet seed. The chitosan solution was diluted to 1:5, 1:10, 1:15, 1:19, and 1:25 and used to soak for 3, 6, and 9 h. The dilution of the chitosan solution tested was 1:19, and soaking time of 6 h showed maximum germination of 91% compared to the control of 83% (Sarathchandra and Jaj 2004).

Chitosan oligomer solution with molecular weight of 2 kDa was also studied to treat cotton seeds at concentration of 0.05%, 0.1%, 0.2%, and 0.3% and soaking time of 15 min. Germination rate of chitosan oligomer–treated seed was from 94% to 95.6% higher than the control (92%), and this difference was significant with $P \leq 0.05$ (Dzung 2004a,b).

Chitosan was also used to produce synseeds (artificial seeds) of *Cymbidium* orchid. Protocorm-like bodies (PLBs) of *Cymbidium* were coated in sodium alginate and external chitosan coating. At the same sugar concentration, a higher concentration of chitosan showed a higher survival rate of the synseeds but lower shoot formation. It was found that sucrose concentration of 60 g/L and coated by 10 g/L chitosan were optimal for transplanting *Cymbidium* synseeds into greenhouse (Nhut et al. 2005).

A review of the mentioned published papers shows that the germination stimulating activity of chitosan and their derivatives is strongly dependent on molecular weight, concentration, soaking time, and various plants. Therefore, to apply the technique of seed coating, we have to test carefully all the mentioned conditions.

42.1.5 Improving Mineral Nutrient Uptake of Plants

Chitosan, chitosan oligomer, and salicyden-chitosan oligomer were sprayed on leaves of cabbage at a concentration of 25 ppm. The amount of nitrogen in the leaves was analyzed and it was found that accumulation of nitrogen in treated chitosan, chitosan oligomer, and salicyden-chitosan oligomer cabbage was higher than the control from 3.0% to 11.0% (Dzung 2005). Li and Wu (1998) reported that the mineral composition of spoon cabbage was not changed when coating seed with chitosan solution, although the treatment was more effective on the yield of spoon cabbage.

In another research, coffee seedlings were sprayed with chitosan oligomer in greenhouse at concentration from 20 to 80 ppm. Observed results indicated that chitosan oligomer increased in accumulation of mineral composition from 3.3% to 13.6% compared to the control, and the difference was significant at $P \leq 0.01$ (Nam 2008). At concentration of 60 ppm, chitosan oligomer was the most effective on accumulation of mineral composition in coffee leaves. Effect of chitosan oligomer on improving mineral uptake of coffee was also carried out in field, as shown in Table 42.4.

The results shown in Table 42.4 indicated that chitosan oligomer concentration of 50 ppm was the best choice for accumulation of nutrients in coffee leaf. At this concentration, chitosan oligomer increased in 9.49% N, 11.76% P, 0.98% K, 3.77% Ca, and 18.75% Mg compared to control. A special interest is the significant increase in content of N and Mg to be directly proportional to increase in content of chlorophyll.

To evaluate effect of chitosan application on soil fertility, the samples of soil before and after cultivation of soybean were collected and analyzed. The results indicated that mineral composition in soil

TABLE 42.4
Effects of Chitosan Oligomer Concentration and Times of Foliar on Chemical Nutrients in Coffee Leaf

Times of Foliar	Concentration	N%	P%	K%	Ca%	Mg%
	0 ppm	2.95[b]	0.16[b]	2.01[a]	1.03[a]	0.30[a]
I	30 ppm	3.16[a]	0.18[a]	2.02[a]	1.06[a]	0.30[a]
	50 ppm	3.02[a]	0.18[a]	2.02[a]	1.01[a]	0.35[b]
	0 ppm	2.96[b]	0.17[b]	2.05[a]	1.03[a]	0.30[a]
II	30 ppm	3.23[a]	0.19[a]	2.06[a]	1.04[a]	0.35[b]
	50 ppm	3.34[a]	0.19[a]	2.08[a]	1.13[a]	0.39[b]
	0 ppm	2.93[b]	0.17[b]	2.05[a]	1.12[a]	0.35[a]
III	30 ppm	3.16[a]	0.18[a]	2.07[a]	1.16[a]	0.38[b]
	50 ppm	3.34[a]	0.19[a]	2.07[a]	1.15[a]	0.41[b]

[a,b,c] Significant difference between the treatments at 5% level according to Duncan's Multiple Range Test.

seemed to be unchanged before and after application. The content of nitrogen and potassium increased with the application of chitosan, and there was little increase in the phosphate content (Dzung 2007).

42.1.6 Reducing Stress of Plants

It is known that plants can be stressed by heavy metals, drought, wind, salt, and heat, particularly, with global climate change. Recently, chitosan and their derivatives have been reported to reduce stress in plants. As chelating capacity of chitosan for heavy metals, degraded chitosan by gamma rays was used to reduce stress of rice with vanadium (Tham et al. 2001). Using degraded chitosan with concentration of 200 ppm in hydroponic solution supplemented 10 μg/mL vanadium reduced fourfold accumulation of vanadium in shoot, twofold in root of wheat, and over sevenfold in shoot of rice. Supplement of the degraded chitosan at concentration of 100 ppm into hydroponic solution containing 10 ppm vanadium not only suppressed toxicity of vanadium but also introduced growth promotion. Dry weight of seedlings of rice increased by 13% in comparison to the control.

Foliar application of chitosan reduced transpiration of plant by inducing closure of plant stomata (Bittelli et al. 2001). It was found that the foliar application reduced water use of pepper by 26%–43% while maintaining biomass and yield of pepper in growth chamber as well as field condition. Reducing water use by chitosan foliar application is the best way in helping plants against drought.

Limpanavech et al. (2008) also demonstrated that chitosan increased significantly the number of vascular bundles containing silica cells. This affects silica uptake and silica metabolism in the orchid and plays a key role in heat, wind, and salt stress tolerance. Chitosan foliar is also applied for frost protection in agriculture (Rinaudo 2006).

Chitosan oligomer sprayed on the leaf of coffee seedlings was also found to reduce transpiration of the leaves by 11%, and application of chitosan oligomer increased the drought resistance of coffee seedlings (Nam 2008 unpublished).

42.2 CHITIN, CHITOSAN, AND THEIR DERIVATIVES ENHANCE THE GROWTH AND YIELD OF CROPS

It has been known that both bioactivities of chitosan and their derivatives as induction of defensive system of plants and plant-growth promotion play key roles in application of chitosan for agriculture.

Depolymerized chitin, carboxymethyl, and hydroxyethyl-chitin were used to coat soybean seeds before plantation in the field. It was reported that dry weight of the plants increased by 8% and the crop yield also increased by 12% in comparison to the control (Hirano 1996). Potato tubes were also coated with chitin and chitosan powder and the yield of potato increased up to 50% (Hirano 1996). Wheat seeds were also coated with chitosan and vigor index of wheat seedlings increased by 20%–30% compared to the control, and suitable concentration for chitosan treatment was 4 mg/mL (Reddy et al. 1999).

Effects of chitosan application on the growth and yield of some crops such as soybean, upland rice, mini-tomato, lettuce, and radish were carried out by mixing chitosan 100 kDa at 0.1%–0.5% of soil. It was found to be strongly effective to the growth of the plants. Chitosan application at concentration of 0.5% was suitable for soybean and upland rice, but 0.1% was more effective on lettuce and mini-tomato. The leaf area of lettuce increased by 50%–60% compared to the control after three times of foliar application at concentration of 0.1% and the leaf area of radish also increased up to 100% at concentration of 0.5% (Chibu 2001).

Degraded chitosan by irradiation of gamma rays supplemented in hydroponic solutions at concentration of 100 and 200 ppm. It was found that the chitosan stimulated the growth of rice up to 24% and wheat up to 40% in comparison to the control (Tham et al. 2001). Chitosan oligomer produced by gamma irradiation was used to supplement in the hydroponic solution for cultivation of barley and soybean. Chitosan oligomer with molecular weight of 1–3 kDa was found to exhibit the highest activity for the growth of plants; fresh biomass of barley and soybean increased by 15%–30%, and concentration of 20 ppm was optimal for the growth of plants. The chitosan oligomer also increased the yield of soybean by 15.5% (Luan et al. 2006). Rapeseed was also coated with non-irradiated and irradiated chitosan in amounts of 0.1, 0.2, and 0.4 g/kg seed. It was observed that chitosan coating at 0.1 g/kg seed was suitable for the growth promotion of rapeseed. The results showed that biomass of shoot increased by 16%–22%, biomass of roots also increased by 51%–65% (Chmielewski et al. 2007).

Chitosan solution was also used to coat seeds of pearl millet and foliar spray. Chitosan was effective on the growth of pearl millet. Chitosan treatment increased the height of pearl millet from 81.6 cm (control) to 115.7 cm (treatment). The number of tillers, number of ear heads, and 1000 seed weight also increased significantly in the treated plants compared to the untreated plants. It was found that there was 19% increase in 1000 seed weight and 50% in number of ear heads (Sarathchandra and Jaj 2004).

Flower plants have been treated with chitosan and chitosan derivatives widely in Japan, Thailand, Mexico, China, and Korea. Some species of flowers such as *E. grandiflorum,* orchid *Dendrobium*, *Gladiolus*, and *Gerbera* have been studied for their treatment with chitosan and their derivatives. Chitosan not only showed plant-growth promotion such as increase in height, leaf area, and biomass but also effected on development of the plants such as stimulation of flower, increase in number of flowers, pigment, and vase life.

Gerbera (*Gerbera jamesonii*) was sprayed with chitosan 80% DD at concentration of 10, 20, 40, and 60 ppm. Results showed that stem length of *Gerbera* increased from 28.28 (control) to 30.68 cm (at 40 ppm). The leaf length was also increased from 7.39 (control) to 10.75 cm (at 40 ppm). But increase in the number of flowers per bush was only optimal at 60 ppm. At this concentration, the number of flowers increased from 2.84 unit (control) to 4.98 unit (at 60 ppm). Therefore, it was found that chitosan at 40 ppm was suitable for growth of *Gerbera*, and chitosan at 60 ppm was effective to develop *Gerbera* (Wanichpongpan et al. 2001).

E. grandiflorum was treated with chitosan solution and monosaccharides to enhance bud growth and petal pigmentation. Treatment with chitosan promoted processes in developing flower bud and enhanced the accumulation of anthocyanin in petals in vitro to increase the quality of the flowers (Uddin et al. 2004).

Chitosan and hot water were also used to treat *gladiolus* (*Gladiolus* spp). *Gladiolus* corms were dipped in chitosan solution at 1.0% and 1.5% for 30, 60, and 120 min before plantation. Chitosan treatment was strongly effective to increase the number of leaves, number of flowers per spike, vase life, and number of cormlets. The differences of mentioned data were significant at $P \leq 0.05$. Complex treatment of chitosan and hot water showed more effective activity than the chitosan only. *Gladiolus*

treated with 1.5% chitosan increased the number of flowers by 2–7 and vase life also extended for 3 days (Ramos-Garcia et al. 2009).

Effects of DD%, molecular weight, and concentration of chitosan on floral production of *Dendrobium* orchid were also studied (Limpanavech et al. 2008). Chitosan O-80 (45 kDa, >90% DD) at concentrations of 1, 10, 50, and 100 ppm was found to be the most effective on inducing early flowering and increasing in inflorescence number and flowers per inflorescence compared to the control. Treatment of chitosan O-80 at 10 ppm increased inflorescence number per plant from 5.50 (control) to 7.75 (treatment) and flowers per inflorescence from 5.66 to 6.89.

In Vietnam, a lot of crops have been applied with chitosan and their derivatives by coating seed or foliar in fields such as soybean, peanut, cabbage, pea, kohlrabi, rice, cotton, and coffee; the results are shown in Table 42.5.

Soybean seeds were coated with chitosan solution at 0.05%, 0.10%, 0.2%, and 0.30% grown stronger than the control by 25%, and the yield also increased up to 36%. In addition, the rate of rust disease reduced significantly (Dzung and Thang 2002). Effect of spraying chitosan 1–2 kDa at 20–50 ppm on the growth and yield of peanuts was studied in field. It was found that the growth, dry biomass, and effective branches of the treated peanuts were higher than the control, as shown in Table 42.6.

Comparison of chitosan oligomer with some other foliars such as Komix and Song Gianh foliar was also carried out in field. Results showed that Komix and Song Gianh foliar increased only by 2.82%–6.45% while chitosan oligomer increased up to 47.98% (Dzung 2004a,b).

Rice is one of the most important crops of Vietnam and annual rice export is about 4–5 million tons. Foliar application of chitosan oligomer on rice was carried out at Rice Research Institute of Mekong Delta, Vietnam. Chitosan application was compared to some foliars such as Bioted-603 and HVP-401 (Dzung 2007). Results showed that chitosan oligomer increased significantly in plump grain/ear, crop yield up to 20% compared to the control and higher than the other foliar as Bioted-603 and HVP-401. Nitar et al. (2004) also studied the effect of chitosan oligomer at 20 ppm on rice in

TABLE 42.5
Effect of Chitosan Oligomer Concentration on Increasing Yield of Some Crops by Foliar Application

Plants	0 ppm	20 ppm	30 ppm	40 ppm	50 ppm	80 ppm	References
Peanut	0.00	19.34	22.62	40.65	24.57	—	Dzung (2004a,b)
Soybean	0.00	11.88	16.43	22.3	19.95	—	Dzung (2005)
Kohlrabi	0.00	9.87	11.70	13.10	12.10	22.86	Dzung (2005)
Cabbage	0.00	32.60	35.86	30.43	25.00		Dzung (2005)
Green pea	0.00	11.48	15.02	16.63	15.10	—	Dzung (2005)
Rice	0.00	10.58	—	6.55	7.36	13.86	Dzung (2006, 2007)
Cotton	0.00	—	—	48.60	—	—	Dzung (2007)

TABLE 42.6
Effect of Chitosan Concentration on the Growth and Yield of Peanut

Concentration Observed Data	0 ppm	20 ppm	30 ppm	40 ppm	50 ppm
Growth rate (cm/day)	0.630^{a}	0.709^{a}	0.715^{a}	0.808^{b}	0.739^{b}
Dry biomass (g/plant)	72.89^{a}	76.99^{a}	95.49^{b}	97.21^{b}	8213^{c}
Effective branches/plant	7.68^{a}	8.20^{b}	8.30^{b}	8.20^{b}	7.71^{a}
Number of nodules/five plants	205.33^{a}	194.66^{a}	197.33^{a}	308.33^{b}	337.66^{b}
Yield% (comparison in control)	0.00	19.34	22.62	40.65	24.57

^{a,b,c} Significant difference between the treatments at 5% level according to Duncan's Multiple Range Test.

Myanmar by soaking rice seeds for 24 h. Chitosan oligomer application increased height of rice from 15 up to 22.5 in. The production yield of treated rice was 1.6 times higher than the control.

In another place, Dzung and Thuoc (2006) also studied effect of chitosan oligomer on growth and crop yield of rice. The experiment was carried out in Ea Kar district, Dak Lak province in 2003. The yield of treated rice at 40 ppm chitosan foliar application increased by 12.35% compared to the control and higher than the Grow and AC foliar. The leaf of treated rice seemed greener than the control before harvesting.

Chitosan foliar application also stimulated the growth of cotton on field. The height of cotton was significant and 10 cm higher than the control. Chitosan solution at 40 ppm increased the production yield of cotton by 15%–40% (Dzung 2007).

Effect of chitosan oligomer on the growth and development of cotton by coating seed was carried out in 2004 (Dzung 2004). Cotton seeds were coated with chitosan oligomer at 0.05%, 0.10%, 0.20%, and 0.30% for 30 min and then plantated. The growth data shown in Table 42.7 indicated that chitosan oligomer stimulated seed germination, the average height of cotton enhanced from 81.72 (control) up to 95.38 cm (0.2% treatment). Chitosan solution at 0.2% was the best choice to coat the cotton seeds, and the number of fruits/plant was the highest and significant in comparison with the control.

Chitosan is a natural product that is nontoxic, biodegradable, and eco-friendly, and is thus an ideal product for producing safe vegetables such as cabbage, spoon cabbage, kohlrabi, green pea, lettuce, and spinach. Li and Wu (1998) used chitosan with various DD% to coat seeds and supplement in hydroponic nutrients solution of vegetables such as spoon cabbage, spinach, lettuce. It was concluded that the seeds with thicker coats were not stimulated as easily as the thinner coats, and acetic acid could be harmful to seed germination. Therefore, Li suggested that water-soluble chitosan was a better choice. Supplement of chitosan 90% DD and at 150–180 ppm to the hydroponic solution of spoon cabbage cultivation was optimal for increase in the growth of spoon cabbage. The yield increased by 30.6% in comparison with the control.

Chibu (2001) applied chitosan powder with 100 kDa at 0.1% in soil to be optimal for the growth of mini-tomato and lettuce. The leaf area of lettuce increased by 50% compared to the control. Pe-tsai (*Brassica campestris*), a variety of cabbage, was sprayed with chitosan 90% DD at concentration of 2, 4, 6, 8, and 10 g/L. The results demonstrated that chitosan effected strongly on the growth of Pe-tsai. The yield of Pe-tsai increased up to 50% compared to the control when applying chitosan solution at concentration from 2 to 4 g/L. If the plant applied chitosan solution at concentration over 4 g/L, the plant showed phytotoxic symptom and the yield of the plant reduced (Wongroung et al. 2002). It was also found that the optimal concentration of chitosan oligomer foliar was of 25 ppm for cabbage, green pea, and kohlrabi, and the yield of the vegetables increased from 13.1% to 35.86% (Dzung 2003, 2007).

Chitosan and chitosan oligomer were also sprayed on the leaf of coffee in field. Chitosan and chitosan oligomer increased the content of chlorophyll, mineral nutrient uptake, and growth,

TABLE 42.7
Effect of Coating Seeds by Chitosan Oligomer on the Growth and Development of Cotton

Concentration of Chitosan Oligomer	0%	0.05%	0.1%	0.2%	0.3%
Germination rate, %	92.0	95.3	95.6	94.0	95.0
Height, cm	81.72[a]	93.40[b]	93.25[b]	95.38[b]	87.92[b]
Branches/plant	14.29	15.26	15.15	14.70	14.01
Fruits/plant	19.95[a]	20.82[a]	22.13[a]	22.45[b]	21.37[a]

[a,b,c] Significant difference between the treatments at 5% level according to Duncan's Multiple Range Test.

and reduced the rate of fruit fall from 6.71% to 15.25%. It was found that chitosan oligomer was more effective than the chitosan (Toan 2007 unpublished).

42.3 METHODS OF APPLICATION OF CHITIN, CHITOSAN, AND THEIR DERIVATIVES FOR AGRICULTURE

Chitosan and some of its derivatives are easily soluble in water and dilute organic acids such as lactic and acetic acids; therefore, chitosan and its derivatives can be used in various ways such as soil enrichment, foliar spraying, coating seed, supplement in hydroponic, and supplement in plant tissue culture media in which coating seed and foliar spraying are useful. The methods of application for various plants are reviewed in Table 42.8.

It is known that difficulties of chitosan application for agriculture are chitosan standardization and not stable effectiveness, because of bioactivity of chitosan such as growth promotion, enhancing crop yield, and antifungal activity are dependent on DD%, molecular weight, concentration of chitosan, and plants. Table 42.9 reviews methods for using chitosan; for promoting the application of chitosan for agriculture; and for contributing to the development of sustainable agriculture, optimal concentration, molecular weight, and DD%. Based on researches, it is concluded that

1. Suitable concentration for coating seeds is from 0.2% to 1.0%.
2. Chitosan powder for soil enrichment is 0.1%–0.5%.
3. Chitosan with M_w 20–45 kDa for foliar spraying is 0.1%–0.2%.
4. Chitosan oligomer with M_w of 2–3 kDa for foliar spraying is 20–50 ppm for the growth promotion and higher for development.
5. Chitosan oligomer with M_w of 1–3 kDa for plant tissue culture is 15 ppm and the M_w is higher using higher concentration.

TABLE 42.8
Useful Methods of Chitosan Application for Plants

Methods of Application	Materials	For Plants	References
Soil enrichment	Powder	Potato, soybean, lettuce, mini-tomato, spinach	Chibu and Shibayama (2000), Hirano (1996)
	Solution	Black pepper	Dzung et al. (2006)
Foliar spraying	Solution	Peanut, soybean, cabbage, green pea, rice, maize, coffee, black pepper, cotton, lettuce, spinach, *Dendrobium* orchid, *Eustoma*, pepper, *Gladiolus*	Dzung and Thang 2004, Dzung (2004a,b, 2005, 2006, 2007), Dzung et al. 2006, Limpanavech et al. (2008), Uddin et al. (2004), Bittelli et al. (2001), Ramos-Garcia et al. (2009), Dzung and Thang (2004)
Seed coating		Soybean, cotton, pearl millet, cucumber, pumpkin, chili, cabbage, wheat, rice, rapeseed, *Cymbidium* orchid	Dzung (2002, 2005), Reddy et al. (1999), Sarathchandra and Jaj (2004), Nitar et al. (2004), Chandrkrachang (2002), Chmielewski et al. (2007), Nhut et al. (2005)
Supplement in hydroponic	Solution	Rice, wheat, peanut, barley, soybean, spoon cabbage	Tham et al. (2001), Li (1998), Luan et al. (2006)
Supplement in plant tissue culture medium	Solution	*Chrysanthemum, Lisianthus, Limonium, Dendrobium, Taxus, Rubia*, carrot	Luan (2002, 2005), Nge et al. (2006), Li and Tao (2009), Vasconsuelo et al. (2004), Kitto et al. (1991)

TABLE 42.9
Concentration and Properties of Chitin, Chitosan, and Their Derivatives Applied for Agriculture

Materials	M_w	DD%	Concentration	Methods	For Plants	References
Chitosan	—	—	4–8 mg/mL	Coating seed	Wheat	Reddy et al. (1999)
CM-Chitosan	—	90	1.0%–1.5%	Coating seed	Spoon cabbage, amaranthus, spinach, lettuce	Li (1998)
Depolymerized chitin, CM-chitin	—	—	—	Coating seed	Soybean	Hirano (1996)
Chitosan powder	100 kDa	—	0.1% 0.5%	Application to soil	Soybean, upland rice, lettuce, tomato	Chibu (2001)
Chitosan	20 kDa	76%	0.1%	Spraying	Pepper	Bittelli et al. (2001)
Chitosan oligomer	2 kDa	75%	30–40 ppm	Spraying	Peanut, soybean, kohlrabi, rice, cotton, green pea, coffee, cabbage	Dzung (2004a,b, 2005, 2006, 2007)
Chitosan	—	—	0.2%	Spraying	Pearl millet	Sarathchandra and Jaj (2004)
Chitosan	45 kDa	90%	10–50 ppm	Spraying	*Dendrobium* orchid	Limpanavech et al. (2008)
Chitosan oligomer	1–3 kDa	—	20 ppm	Hydroponic solution	Soybean, barley	Luan et al. (2006)
Chitosan oligomer	1 kDa 16 kDa	— —	15 ppm 30–100 ppm	Plant tissue culture in vitro	*Dendrobium* orchid *Chrysanthemum, Lisianthus, Limonium*	Nge et al. (2006), Luan (2005)

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Index

E

F

Q

R

S

W

X

Y

Z

Food Science & Technology

Chitin, Chitosan, Oligosaccharides and Their Derivatives

Biological Activities and Applications

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