BIOMATERIALS for DELIVERY and TARGETING of PROTEINS and NUCLEIC ACIDS

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EDITED BY Ram I. Mahato



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Dedication

I dedicate this book to Vivek, Kalika, and my wife Subhashini for all their love and support; and to my students and mentors who have always helped me in my quest for learning and in achieving higher goals.

Preface

Progress in biotechnology has created many opportunities for the development of protein- and nucleic acid-based therapeutics for the treatment of genetic and acquired diseases. There are numerous advanced books on polymer synthesis, drug delivery, oligonucleotides, and gene therapy, but there is an urgent need for a textbook for newcomers to this field including graduate students and young scientists, who have little understanding of the field. This book is expected to serve as a textbook and/or reference text for graduate courses in biomaterials and delivery of proteins and nucleic acids.

Among scientists with different expertise, effective protein and nucleic acidbased therapeutics require a multi-disciplinary approach, such as molecular and cell biology, biochemistry, biophysics, polymer chemistry, colloid science, pharmaceutics, and medicine. Significant progress has been made in the use of biomaterials and polymeric carriers for the delivery of proteins, peptides, and nucleic acids (including plasmid DNA, antisense and antigene oligonucleotides, and siRNA). In addition to their use as carriers, polymers are finding increased use in polymer therapeutics, whereby the conjugated polymeric carriers usefully alter the properties of the protein or the nucleic acid. Liposomes have also been proven useful for delivery of proteins and nucleic acids. With this end in mind, I have organized this book to reflect various aspects of the field namely:

- Use of polymers --structure, properties, synthesis and characterization
- Crosslinking and PEGylation of proteins
- Biocompatibility and biological barriers
- In vivo and subcellular fate
- Stability and formulation aspects of proteins and nucleic acids
- Micro- and nano-particulate and liposomal delivery systems
- Drug resistance and transporters
- Protein transduction domain peptides
- Antisense and antigene approaches
- Artificial nucleic acid chaperones
- Basic elements of nonviral gene therapy

This book is written by international experts and leaders in their respective fields of knowledge. We have attempted to convey both an introductory understanding as well as latest developments in the field so that this book will be useful for both novice students and practicing scientists. We hope that this book will stimulate deeper understanding and interest in this integrated field, from people with diverse expertise and backgrounds.

About the Editor

Ram I. Mahato is an assistant professor of pharmaceutics and drug delivery at the Departments of Pharmaceutical Sciences and Biomedical Engineering, University of Tennessee, Memphis. Dr. Mahato has served as a research assistant professor at the University of Utah; senior scientist at Valentis, Inc. (formerly, GeneMedicine, Inc.); and as a postdoctoral fellow at the University of Southern California, Washington University, and Kyoto University. He received a Ph.D. in pharmaceutics and drug delivery from the University of Strathclyde (Glasgow, U.K.) in 1992, and B.S. in pharmaceutics from China Pharmaceutical University (Nanjing, China) in 1989.

Dr. Mahato has published more than 60 papers and book chapters, obtained one U.S. patent, and edited theme issues on nucleic acid delivery for the *Journal of Drug Targeting* and the *Advanced Drug Delivery Reviews*. In July 1992, he edited a book entitled *Pharmaceutical Perspectives of Nucleic Acid-Based Therapeutics*. He is a member of the editorial boards of the *Journal of Drug Targeting* and *Expert Opinions on Drug Delivery*. He is a frequent reviewer for the National Institutes of Health and several international journals.

Dr. Mahato's present research interests include design of novel polymers, cationic lipids, and lipopolymers for nonviral delivery of oligonucleotides and genes, use of modified adenovirus for gene delivery to primary cells, pharmacokinetics and biodistribution of nucleic acids, and protein delivery.

Contributors

Shikha P. Barman

Zycos, Inc. Lexington, Massachusetts

Jean-Pierre Benoît

INSERM U646 Faculty of Pharmacy University of Angers Angers, France

Joon Sig Choi

Department of Biochemistry Chungnam National University Daejeon, Korea

Abraham J. Domb

Department of Medicinal Chemistry and Natural Products School of Pharmacy, Faculty of Medicine Hebrew University of Jerusalem Jerusalem, Israel

Dagmar Fischer

University of Marburg Marburg, Germany

Sven Frokjaer

The Danish University of Pharmaceutical Sciences Copenhagen, Denmark

Hamidreza Ghandehari

University of Maryland School of Pharmacy Department of Pharmaceutical Sciences Baltimore, Maryland

Mark Gumbleton

Pharmaceutical Cell Biology Welsh School of Pharmacy Cardiff University Cardiff, Wales

Ramareddy V. Guntaka

Department of Molecular Sciences University of Tennessee Health Science Center Memphis, Tennessee

Mary Lynne Hedley

Zycos, Inc. Lexington, Massachusetts

Kenneth D. Hinds

Centocor, Inc. Radnor, Pennsylvania

Lars Hovgaard

The Danish University of Pharmaceutical Sciences Copenhagen, Denmark

Kang Moo Huh

Department of Pharmaceutics and Biomedical Engineering Purdue University School of Pharmacy West Lafayette, Indiana

Young Jin Kim

Department of Polymer Science and Engineering Chungnam National University Daejeon, South Korea

Sung Wan Kim

Center for Controlled Chemical Delivery Department of Pharmaceutics and Pharmaceutical Chemistry University of Utah Salt Lake City, Utah

Dagmar Klein

Diabetes Research Institute University of Miami School of Medicine Miami, Florida

Minhyung Lee

Clinical Research Center Department of Molecular Gene Medicine Inha University Medical School Inchon, Korea

Sang Cheon Lee

Department of Pharmaceutics and Biomedical Engineering Purdue University School of Pharmacy West Lafayette, Indiana

Zheng-Rong Lu

Department of Pharmaceutics and Pharmaceutical Chemistry University of Utah Salt Lake City, Utah

Ram I. Mahato

Department of Pharmaceutical Sciences University of Tennessee Health Science Center Memphis, Tennessee

Atsushi Maruyama

Institute of Materials Chemistry and Engineering Kyushu University Fukuoka, Japan

Bernd Meibohm

Department of Pharmaceutical Sciences University of Tennessee Health Science Center Memphis, Tennessee

Tamara Minko

Department of Pharmaceutics The State University of New Jersey Piscataway, New Jersey

Anjan Nan

University of Maryland School of Pharmacy Department of Pharmaceutical Sciences Baltimore, Maryland

Yadollah Omidi

Pharmaceutical Cell Biology Welsh School of Pharmacy Cardiff University Cardiff, Wales

Tooru Ooya

School of Materials of Science Japan Advanced Institute Science and Technology Ishikawa, Japan

David Oupický

Department of Pharmaceutical Sciences Wayne State University Detroit, Michigan

Jong-Sang Park

Department of Chemistry Seoul National University Seoul, Korea

Kinam Park

Department of Pharmaceutics and Biomedical Engineering Purdue University School of Pharmacy West Lafayette, Indiana

Catherine Passirani

INSERM U646 Faculty of Pharmacy University of Angers Angers, France

Ricardo L. Pastori

Diabetes Research Institute University of Miami School of Medicine Miami, Florida

Rajendra P. Pawar

Department of Medicinal Chemistry and Natural Products School of Pharmacy, Faculty of Medicine Hebrew University of Jerusalem Jerusalem, Israel

Melina M. Ribeiro

Diabetes Research Institute University of Miami School of Medicine Miami, Florida

Camillo Ricordi

Diabetes Research Institute University of Miami School of Medicine Miami, Florida

Andreas Schätzlein

Department of Medical Oncology Beatson Laboratories University of Glasgow Glasgow, Scotland

Ikumi Tamai

Faculty of Pharmaceutical Sciences Tokyo University of Science Chiba, Japan

Vladimir Torchilin

Department of Pharmaceutical Sciences Northeastern University Boston, Massachusetts

Marco van de Weert

The Danish University of Pharmaceutical Sciences Copenhagen, Denmark

Zhaoyang Ye

Department of Pharmaceutical Sciences University of Tennessee Health Science Center Memphis, Tennessee

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1 Structure, Properties, and Characterization of Polymeric Biomaterials

Anjan Nan and Hamidreza Ghandehari

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1.1 INTRODUCTION

Biomaterials is a term used to indicate materials that constitute the basic framework of medical implants, extracorporeal devices and disposables utilized in medicine, surgery, dentistry, veterinary, as well as in other aspects of medical care. Biomaterials can be natural or synthetic in origin. They are used in a variety of ways from being carrier molecules for delivery of bioactive agents (small molecular weight drugs, proteins, peptides, oligonucleotides, and genes) or as whole or part of systems that augment or replace tissues or organs. The term "biomaterials" make them unique from other classes of materials in that they are required to meet special biocompatibility criteria (as discussed in Chapter 6) for acceptance in the biological system. Polymeric constructs are a major component of biomaterials.

Polymers are macromolecules consisting of multiple repeating units or monomer residues linked together usually by covalent linkages. End-groups are the structural units that terminate polymer chains. Polymers containing reactive end-groups to allow for further chemical modification are called telechelic polymers. The monomers in polymeric systems can be linked together in various ways to give rise to linear chains, branched or three-dimensional cross-linked networks (Figure 1.1). A linear polymer has no branching. A typical example is poly(ethylene glycol) (PEG) (Table 1.1). Linear polymers can have pendent groups associated with them. Example of linear polymeric systems with pendent side-groups are N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers (Table 1.1). Branched polymers are those in which the molecules have been formed by branching as opposed to a linear polymer. A polymer with a high degree of three-dimensional tree-like branching starting from a core is called a dendrimer. Three-dimensional polymers are also formed



FIGURE 1.1 Various types of polymeric architectures: (a) linear with end-chain; (b) linear with side-chain; (c) branched; (d) cross-linked and (e) dendritic. Drugs can be attached to or dispersed in these polymers.

TABLE 1.1 General and specific structures of some commonly used polymers in drug delivery

Synthetic biodegradable polymers

Poly(ortho ester)

Polyphosphoesters

Polyanhydrides

Polyesters

Methyl vinyl ether and maleic anhydride copolymer

Polycarbonates

Poly(amino acid)s

Synthetic non-degradable polymers

Silicone elastomers

Poly[ethylene-co-(vinyl acetate)]

Acrylic polymers

Poly ethylene oxide/Poly ethylene glycol



N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer



Natural and protein based polymers

i. Saccharides Chitosan





Dextran

ii. Collagen (typical amino acid sequence)

 $[(Gly)-X-Y]_n (X \text{ is often Proline},$ Y is often hydroxyproline)



iii. Cellulose derivatives

Genetically engineered polymers

Elastin-like polymers

Typical silk-like polymers Silk elastin-like block copolymers

[Poly(alanylglycine)]

 $[VPGXG]_n (X = any amino$ acid except proline) $[(GAGAGS)_9 GAAVTGRGDSPASAAGY]_n]$ $[(GAGAGS)_m(GVGXP)_n]_o (X = any amino$ acid except proline) $-(AG)_mX_n (X = any amino acid)$



FIGURE 1.2 Various types of copolymers based on monomeric arrangements: (a) random; (b) alternating; (c) block and (d) graft. These structures comprise of A and B as two representative comonomers. Polymers can also be synthesized to contain more than two types of comonomers.

by physical or chemical cross-linking of polymer strands to form a network. A typical example of such a structure is a hydrogel. Hydrogels are threedimensional polymeric networks that swell in water but do not dissolve.

Polymers can be classified based on the general composition of the monomeric components as homochain polymers and heterochain polymers. In homochain polymers the polymer chain or backbone consists of a single type of atom. Primarily this is carbon with other atoms or groups of atoms attached. Heterochain polymers such as polyethers or polyesters contain more than one atom type in the backbone. The degree of polymerization refers to the total number of structural monomeric units including end groups and is thus related to the chain length and the molecular weight.

Another way to classify polymers is based on the constituents of the chain. A polymer prepared from a single monomer is called a homopolymer. If two or more types of monomers are employed it is called a copolymer. Depending on the arrangement of monomers, various types of copolymers can be identified. The monomeric units may be randomly distributed (random copolymer), in alternating fashion (alternating copolymer), or in blocks (block copolymer). A graft copolymer consists of one polymer grafted to the backbone of the other. Using A and B to denote the two different monomers, various types of copolymers are depicted in Figure 1.2. Polymers are conventionally classified based on two major methods of synthesis namely chain polymerization and step-growth polymerization. Details of such classification and synthesis are discussed in the latter chapters of this book.

The following section provides the reader with a general overview of the different types of polymers that are used primarily in biomedical applications with an emphasis on drug delivery. For a more detailed classification and properties of polymers the readers are referred to the *Encyclopedia of Polymer Science and Technology*.¹ Table 1.1 lists the structures of commonly used polymers in drug delivery as discussed in the following sections. The list is not comprehensive and intends to introduce some of the polymers used in this field.

1.2 SYNTHETIC POLYMERS

1.2.1 BIODEGRADABLE POLYMERS

Biodegradable polymers break down to smaller fragments due to pH change or enzymatic hydrolysis. The following section reviews the characteristics of some commonly used families of biodegradable polymers used in medicine and drug delivery.

1.2.1.1 Poly(ortho ester)s

Poly(ortho ester)s were pioneered for biomedical applications in the 1970s by Choi and Heller at Alza Corporation (Palo Alto, CA) under the name of Alzamer[®]. These polymers have been categorized into three major families, namely I, II, and III, based on their methods of synthesis and polymer degradation mechanisms.² These polymers usually undergo hydrolysis in an aqueous environment to produce a diol and a lactone, which rapidly converts to γ -hydroxybutyric acid. The γ -hydroxybutyric acid autocatalyzes the hydrolysis reaction. The rate and extent of degradation by autocatalysis can be controlled by incorporation of a basic moiety such as sodium bicarbonate. Poly(ortho ester)s have been used for a number of drug delivery applications.³ For example poly(ortho ester) I has been used for delivery of the narcotic antagonist Naltrexone,⁴ while poly(ortho ester) III has been used for delivery of 5-fluorouracil as an adjunct for glaucoma filtration surgery. Several modifications of poly(ortho ester)s have been introduced over the years, which allow synthesis under milder conditions, or polymers, which are ointment at room temperature rendering them suitable for topical and periodontal applications.5

1.2.1.2 Poly(phosphoester)s

This family of polymers is comprised of the polyphosphates, polyphosphonates, and polyphosphites, which are synthesized by altering the functional group on the main chain (\mathbf{R}) or the side chain (\mathbf{R}') of the general polymer backbone (Table 1.1). Accordingly the physicochemical properties of these polymers can be changed. Also by modifying the backbone of these polymers, controlled biodegradation can be achieved. These polymers can be synthesized with high molecular weights of over 100,000 resulting in good mechanical properties. The hydrolytic breakdown products of these polymers are phosphates, alcohols,

and diols which are all potentially nontoxic. The pentavalent phosphorus atom easily allows chemical conjugation of drugs and other molecules to the polymer side chain. Several examples of poly(phosphoester) (PPE)-based drug delivery systems are reported in the literature. PPE microspheres have been used to deliver drugs such as paclitaxel, cisplatin, and lidocaine.⁶ The rate of drug release has been shown to depend on the side chain length of the polymer. Recently PPE-based microspheres were used in the controlled release of plasmid DNA with an encapsulation efficiency as high as 88–95%.⁶

1.2.1.3 Polyanhydrides

The anhydride linkage in polymer chains is highly susceptible to hydrolysis. As a result several properties are required for preparing stable anhydride devices. Of particular interest in drug delivery are polymers where hydrolysis results in surface erosion. For example, the anhydride copolymer of sebacic acid and carboxyphenoxypropane resists water penetration due to the hydrophobic carboxyphenoxypropane residue, yet degrades into low molecular weight fractions at the water/polymer interface owing to the presence of anhydride bond. By appropriately selecting the copolymerization ratios we can modulate the hydrophobicity of the matrix and thus the degradation rate. Polyanhydrides can be manufactured by varying the R_1 and R_2 functional groups in the backbone (Table 1.1) as aliphatic or aromatic homopolymers or copolymers as well as cross-linked or branched polymers. A number of recent reviews discuss various applications of polyanhydrides.⁷ A typical example is the polyanhydride implant (Septacin[®], Abbott Laboratories, Abbott Park, IL, USA) containing gentamicin sulfate developed for sustained local delivery in the treatment of osteomyelitis.⁸ Polyanhydride has also been used for delivery of camptothecin for the treatment of gliosarcoma.⁹

1.2.1.4 Polyesters

This class of polymers include the most widely used degradable polymers namely poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and copolymers of lactide/glycolide such as poly(D,L-lactide-*co*-glycolide) (PLGA). These polymers are classified based on the different substituents (R_1 , R_2 : Table 1.1) on the backbone with a primary ester linkage. Polyesters are of great interest in drug delivery and tissue engineering because of several important characteristics. They break down to naturally occurring metabolites, e.g., lactic acid and glycolic acid. The degradation of these polymers is mediated simply by water and variation in pH. The PLGA copolymers have been well established in terms of their physicochemical properties and safety in humans.^{10,11} A modification of the polyesters, namely poly(ε -caprolactone), is a biodegradable polymer which has found useful applications in drug delivery.¹² These polymers degrade slower than PLGA and are suitable for sustained release formulations. Copolymers of lactide and lysine comonomers which have primary amine

functionality have been used for coupling to peptides and other functional moieties.¹³

The chemical structures of PLA and PGA are similar except that PLA has a methyl pendant group making it more hydrophobic, and this contributes to differences in their degradation kinetics. As a result, the degradation rate of PLGA depends on the molar ratio of lactide and glycolide present in the polymer. An increase in lactide ratio causes faster degradation due to decreased crystallinity. The polymer characteristics also depend on stereochemistry. The racemic poly(D,L-lactide) (DL-PLA) is less crystalline and has a lower melting point than the two stereoregular polymers, D-PLA and L-PLA. Further, the copolymers of lactide and glycolide are less crystalline and hence more biodegradable than the corresponding homopolymers.^{14,15} Some examples of PLGA-and PLA based drug delivery systems include controlled release of narcotic antagonists and contraceptive steroids from PLA films,^{16,17} Lupron Depot[®] (Abbott Laboratories, Abbott Park, IL, USA), a parenteral sustained release formulation of PLA for prostate cancer, and Nutropin Depot[®] (Genentech, South San Francisco, CA, USA) of PLGA for growth deficiencies.

1.2.1.5 Methyl Vinyl Ether-Maleic Anhydride Copolymer

The key characteristic of these polymers is that degradation occurs by surface erosion thereby offering precise control of dissolution kinetics and drug release.¹⁸ In an aqueous environment the carboxylic acid groups on the polymer become ionized making the polymer water soluble but erosion only occurs at the surface. The degradation is highly pH dependent with increased degradation at higher pH.

1.2.1.6 Polycarbonates

Polycarbonate-based polymers are synthesized in several forms. Poly(trimethylene carbonate) is an aliphatic molecule that degrades under physiological conditions. Aliphatic polycarbonates become extremely soft in the temperature range of 40 to 60°C. Their low softening point and mechanical weakness diminish their use in most applications. Bisphenol-A polycarbonate (poly(BPA carbonate)) is a commercially available polycarbonate (Table 1.1) that is extremely stable and virtually nondegradable under physiological conditions. This polycarbonate exhibits excellent processability, high mechanical strength. and exceptional shatter resistance. In order to decrease the hydrolytic stability of poly(BPA carbonate), the carbonyl oxygen has been replaced by an imino group. This modification produced hydrolytically degradable fibers with strength similar to that of poly(BPA carbonate). Poly(BPA iminocarbonate) was found to be tissue compatible upon subcutaneous implantation in mice and rabbits; however, in an attempt to reduce any potential toxicity, tyrosinederived iminocarbonateamide copolymers were produced.^{19,20} These polymers can be regarded as pseudo poly(amino acid)s that exhibit the biocompatibility
of amino acids while maintaining mechanical strength similar to poly(BPA carbonates).

1.2.1.7 Poly(amino acid)s

These polymers are synthesized from naturally occurring monomers (amino acids) which can degrade into nontoxic components. Although poly(amino acid)s such as polylysines are synthesized by polymerization using conventional methods, these materials are usually immunogenic and exhibit poor mechanical properties.²¹ To overcome such problems, the monomeric amino acid units such as lysine or glutamic acid are modified in their side chains to produce polymers with varying mechanical properties. Copolymers of L-glutamic acid and γ -ethyl L-glutamate with varying ratios of monomers, for example, have been fabricated to deliver a wide variety of drugs with varying release profiles.²² Because of the stability of the peptide bond in water, biodegradation of these polymers occur by dissolution of the intact polymer chains and subsequent enzymatic hydrolysis in the liver or other tissues. Poly(amino acid)s are also synthesized to contain nonpeptide bonds (referred to as pseudo poly(amino acid)s,²⁰ e.g., poly(serine ester)). There are several examples of the use of poly(amino acid)s and pseudo poly(amino acid)s in drug and nucleic acid delivery. Some of the most widely applied polymers include poly[N-(2hydroxyethyl)-L-glutamine] (PHEG),²³ β -poly(2-hydroxyethyl aspartamide) (PHEA),²⁴ poly(glutamic acid),²⁵ poly(aspartic acid),²⁶ and polylysine.²⁷

1.2.2 Nondegradable Polymers

1.2.2.1 Silicone Elastomers

Silicone elastomers have been widely used for a variety of biomedical applications mainly as implants. These polymers have excellent mechanical properties besides being chemically stable. The stability of these polymers towards hydrolysis originates from their hydrophobicity. The polymers are available with a variety of molecular weights, degrees of crosslinking and chemical modification. Although polysiloxanes are mostly used in industrial applications such as lubricants, they are also widely used in biomedical and drug delivery applications. As implants they have received FDA approval for uses such as breast and heart valve prostheses. Several clinical products have been developed for controlled delivery of pharmaceuticals from silicone tubes such as SILASTIC[®] (Dow Corning, Midland, MI, USA) for controlled release of Levonorgestrel, a contraceptive hormone.²⁸ Norplant devices based on silicone elastomers have been used for providing controlled contraception over 5 years in women after forearm implant.²⁹ Silicone polymers have drawn much attention because of the popularity of breast implant devices. Studies in the past 10 years increasingly suggest substantiated risks of these implants such as local inflammatory and scarring reactions, and local infection. Since 1992 FDA has largely controlled the use of such biomedical polymers, although there does not appear to be any evidence of systemic reaction, or abnormalities of the immune system in subjects who have received silicone implants.

1.2.2.2 Poly[ethylene-co-(vinyl acetate)]

These polymers, also referred to as EVAcs, have excellent biocompatibility and are widely used as implants and in topical devices. Typically these polymers contain 40% vinyl acetate. EVAc is a hydrophobic polymer that swells less than 0.8% in water and matrices based on EVAcs are well studied as drug delivery systems for low molecular weight drugs. Protein and macromolecular delivery have also been achieved with these polymers. Some examples of the use of EVAcs in drug delivery include the delivery of pilocarpine to the surface of the eye for treatment of glaucoma which was pioneered by Alza under the name Ocusert Pilo[®]. EVAc is also used in the Progestasert[®] intrauterine device for delivery of contraceptive hormones to the female reproductive tract. Langer and Saltzman have reviewed some applications of EVAc in drug delivery.^{30,31}

1.2.2.3 Acrylic Polymers

Acrylic polymers are a popular choice for controlled release dosage forms.³² Poly(methacrylate) (Eudragit[®], Röhm GmbH & Co. KG, Germany) and poly(methyl methacrylate) (PMMA) are the most commonly used polymers in this class. Eudragits are available in neutral, cationic, or anionic forms and find application in the pharmaceutical industry as an enteric coating material for tablets. They are also used in dermal and transdermal systems. Both Eudragit and PMMA are also used as microsphere or nanoparticulate components for delivery of various drugs.^{33,34}

1.2.2.4 Poly(ethylene oxide)/Poly(ethylene glycol)

Poly(ethylene oxide) (PEO) or poly(ethylene glycol) (PEG) constitutes materials with the general backbone structure of $[-CH_2CH_2O-]$ (Table 1.1). Generally PEG refers to the polymer with molecular weight less than 50,000, while PEO is used for higher molecular weights. PEG is one of the most frequently used water-soluble polymers for biomedical applications because of its high water solubility and chain flexibility. PEG is soluble in a variety of organic solvents depending on its molecular weight. The high degree of hydration and flexibility confers it as protein resistant, biocompatible, and nonimmunogenic. The PEG has been used to prolong the half-life of enzymes conjugated to the molecule thereby decreasing liver uptake. As discussed in Chapter 5, PEGs have been successfully modified at the end groups for the purpose of protein modification. The PEG conjugated adenosine deaminase (ADA), for example, has been approved by the FDA for the treatment of ADA deficiency. Several other proteins such as α -interferon 2a (PEGASYS, Roche Pharmaceuticals, Switzerland), and α -interferon 2b (PEG-INTRON, Schering-Plough, Kenilworth, NJ, USA), are approved by the FDA while others such as hemoglobin (PEG-hemoglobin, Enzon, Bridgewater, NJ), and interleukin 2

(PEG-IL-2, Chiron, Emeryville, CA) are in advanced clinical trials. The PEGs have also been used in drug delivery to increase water-solubility of poorly soluble drugs such as paclitaxel. The long circulating property of PEG is used to improve tumor targeting and accumulation of monoclonal antibody fragments by the passive enhanced permeability and retention (EPR) mechanism.^{35,36} The PEG stabilized liposomal formulations of many drugs have similarly found application in drug delivery.³⁷

1.2.2.5 N-(2-hydroxypropyl)methacrylamide

Pioneered by the work of Kopecek and coworkers, *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers are typically synthesized by free radical copolymerization of HPMA with reactive comonomers resulting in highly water-soluble polymers. By controlling the molecular weight below the threshold of renal excretion the elimination of these polymers from the body after the desired release of bioactive agents can be achieved.^{38,39}

The HPMA based drug delivery systems have been constructed to contain pendent side chains which serve as attachment points for drugs as well as targeting moieties.⁴⁰⁻⁴³ Peptidyl linkers have been particularly attractive because they can be designed to be stable in the blood stream but degradable by lysosomal enzymes leading to intracellular drug release.⁴⁴ The HPMA copolymers containing the tetrapeptide spacer GFLG have been extensively studied for delivery of a large number of anticancer drugs. Several HPMA anticancer drug conjugates such as HPMA-doxorubicin (PK1), HPMA-(galactosamine)-doxorubicin (PK2), HPMA-paclitaxel, HPMA-camptothecin and HPMA-cisplatin are currently in clinical trials for targeted delivery to solid tumors.⁴⁵ The HPMA based drug delivery systems have more recently found application in other areas such as targeting infectious diseases,⁴⁶ radionuclides,⁴⁷ and nuclear targeting.⁴⁸

1.3 NATURAL AND PROTEIN-BASED POLYMERS

1.3.1 POLYSACCHARIDES

Polysaccharides are among the most versatile polymers due to their vast structural diversity and ability to create a variety of linkages between monomer units. Branched polymers are particularly common among polysachharides because of the potential reactivity of all the carbon atoms within the sachharide ring. Two of the most abundantly used saccharides, namely chitin and dextran, are discussed below.

1.3.1.1 Chitin and Chitosan

Chitin is the second most abundant organic compound and is similar to cellulose, except it is composed of *N*-acetylglucosamine in a β -(1,4) linkage. Chitin is readily available and occurs naturally in many insects and marine organisms. It is a popular component of cosmetic and health care products.

A close derivative is chitosan (Table 1.1) consisting of β -(1,4) linked 2-amino-2-deoxyglucopyranose. It is soluble in acidic pH, when the amino group is protonated. The polycationic chitosan is known to enhance drug absorption by modulating the tight junctions of the gastrointestinal epithelial barrier.⁴⁹ Chitosan is biodegradable *in vivo* by glycosidases such as lysozyme.⁵⁰

1.3.1.2 Dextran

Dextran is composed entirely of glucose residues with primarily α -(1,6), and occasionally α -(1,2), α -(1,3), and α -(1,4) linkages for branching. This gives it an open helix conformation (Table 1.1). Dextran has been used as a blood plasma substitute and as carriers of bioactive agents.^{51–54} The compact hydrodynamic volume of dextran due to the hydroxylated glucose units gives it a high renal threshold for excretion (55 to 70 kDa).⁵⁵ Dextran has found a variety of other drug delivery applications including chemical modification with carboxylic groups to reduce clearance rate⁵⁵ or hepatic targeting.⁵⁶

1.3.2 ALBUMIN

Albumin is a major plasma protein constituent accounting for about 55% of the total plasma protein in humans. Albumin is extensively investigated as microspheres in diagnostic nuclear medicine for evaluation of compartmental distribution of drugs in various organs. The favorable features of albumin for drug delivery include its reported biodegradability into natural degradation products, lack of toxicity, and immunogenicity. Albumin has widely been used in drug delivery.^{57–59} Several drugs have been incorporated into various albumin microspheres, whereas human serum albumin (HSA) has been used to deliver a number of drugs and diagnostics. Some common examples include delivery of insulin, 5-fluorouracil, doxorubicin, and mitomycin-C from BSA microspheres or drug conjugates.^{60–62} Preoperative hepatic function has been evaluated using technetium-99m (^{99m}Tc)-diethylenetriaminepentaacetic acid-galactosyl-human serum albumin (Tc-GSA) based scintillation imaging to detect hepatic uptake by the asialoglycoprotein receptors.⁶³

1.3.3 COLLAGEN

Collagen is the major structural protein found in animal tissues. Because of its unique structural properties it has found a variety of biomedical applications such as sutures, wound dressings, facial reconstructive surgery, and as drug delivery vehicles.⁶⁴ It is biocompatible and nontoxic to most tissues and has been used as films for ocular delivery of several ophthalmic drugs such as pilocarpine,⁶⁵ and antibiotics such as tobramycin⁶⁶ with improved bioavailability. Collagen has also been used for protein and peptide delivery. For example, water-soluble protein fractions isolated from bone matrix have been incorporated into collagen matrix and shown to induce bone and cartilage formation *in vivo*.⁶⁷ Osteoinductive devices, comprised of biodegradable

collagen scaffolds and recombinant human bone morphogenetic proteins (rhBMPs), are being currently pursued for local bone induction.⁶⁸

1.3.4 CELLULOSE DERIVATIVES

Cellulose is the most abundantly available organic material; half of all organic carbon in nature is in cellulose form.⁶⁹ Cellulose is a polymer of glucose with the glucose units connected by β -(1,4) linkages (Table 1.1). Cellulose polymer chains are stabilized by hydrogen bonds between adjacent hexose units. Ethyl cellulose is a cellulose ether derivative which is one of the most widely used water-insoluble polymers for coating of solid dosage forms. Besides the predominant use as controlled release barriers, they have also been used as moisture barriers to improve stability of hydrolytically labile drugs or for taste masking purposes. Usually low molecular weight grades are used for coating while high molecular weight grades are used for microencapsulation. Watersoluble cellulose ether derivatives such as hydroxypropyl methylcellulose (HPMC) are often used to modify drug release from delivery systems. It has been shown, for example, that a critical 24% HPMC concentration in a blend of ethyl cellulose and HPMC causes polymer leaching, resulting in pore formation and drug release.^{70,71} Ethyl cellulose and HPMC have been extensively used in a variety of drug delivery models such as matrix preparations and microspheres.72-74

Other naturally occurring polymers include gelatin, casein, and fibrinogen. Various studies have been reported in which these polymers have been tested as drug delivery matrices particularly as microspheres. For example, gelatin microspheres have been used as gastric mucoadhesive drug delivery systems,⁷⁵ casein microspheres for delivery of 5-fluorouracil,⁷⁶ and fibrinogen microspheres for delivery of anticancer drugs like doxorubicin.⁷⁷

1.4 GENETICALLY ENGINEERED AND HYBRID BIOMATERIALS

Genetically engineered polymers consist of repeating peptide sequences, where each repeating unit can be composed of as few as two or as many as hundreds of amino acid residues, and may recur from a few to hundreds of times.⁷⁸ Genetically engineered polymers differ from poly(amino acid)s and sequential polypeptides in that they are synthesized by recombinant techniques. The entire amino acid sequence of genetically engineered polymers is controlled at the DNA-level, leading to polymers with precisely defined, and potentially quite complex, sequences and structures.⁷⁹ Protein-based polymers can be designed to incorporate a variety of functionalities, making them sensitive to physiological stimuli such as pH, temperature, and result in controlled biodegradation, and presentation of informational motifs for cellular and subcellular interactions. The following section briefly reviews some of the genetically engineered polymers that have emerged as novel biomaterials with

potential for drug delivery. For a more detailed discussion of these biomaterials the reader is referred to two recent reviews.^{80,81}

1.4.1 ELASTIN-LIKE POLYMERS

Elastin is an extracellular matrix protein consisting of several repetitive amino acid sequences, including VPGVG, APGVGV, VPGFGVGAG, and VPGG^{82,83} (for amino acid designations, see Section 7). Genetically engineered elastin-like polymers (ELPs) are soluble in aqueous medium below their inverse transition temperature (T_t) ,^{78,84,85} but undergo a sharp reversible phase transition above T_t , leading to desolvation and molecular aggregation to form insoluble elastic fibers *in vivo*.⁸⁶ The loading and release of several ionic probes and drugs, such as Biebrich scarlet red, Naltrexone, Dazmegrel, and Leu-enkephalin, from ELP-based hydrogels composed of poly(GVGVP), poly(GVGIP), and poly(AVGVP) have been studied.

1.4.2 SILK-LIKE POLYMERS

Silks, naturally produced by spiders, are fibrous proteins composed of repetitive sequences of glycine, alanine, and other short chain amino acids having both crystalline and amorphous domains.⁸⁷ The crystalline properties of genetically engineered silk-like polymers (SLPs) have been tailored by the periodic incorporation of amino acids that cannot participate in the β -sheet structure of the polypeptide.⁸⁸ The low aqueous solubility of SLPs has been a barrier to their use in biomedical applications.^{89,90} To enhance the aqueous solubility of recombinant silks, several approaches have been investigated such as inclusion of encoded triggers that regulate self-assembly of the silk, thus improving control over solubility.⁹¹

1.4.3 SILK ELASTIN-LIKE BLOCK COPOLYMERS

Silk elastin-like block copolymers (SELPs) have periodic incorporation of elastin-like blocks within silk domains. This approach increases the solubility of silk-like polymers by reducing their total crystallinity.⁸⁸ SELP solutions of appropriate composition and concentration are liquid at room temperature and can be injected through fine gauge hypodermic needles. *In vivo*, they form hydrogels that are no longer water soluble⁹² leading to controlled release of bioactive agents that might be incorporated in the polymer solution. Release of solutes from these hydrogels is dependent upon the molecular weight, charge, and solubility of the solute, its diffusion through the polymeric matrix, composition of SELP forming hydrogel, and the conditions under which release takes place.^{93–95} The SELPs have been reviewed for their biosynthesis, structures, physicochemical properties, and biological fate.⁹⁶ The SELP based hydrogels have been investigated as vehicles for controlled release of small molecular weight drugs and proteins⁹⁴ as well as plasmid DNA.^{95,97}

Several other novel biomaterials containing genetically engineered motifs have emerged in recent years.⁸⁰ Examples include coiled-coil and leucine-rich

protein polymer domains,⁹⁸ recombinant poly(glutamic acid) polymers,⁹⁹ β -sheet forming polymers,¹⁰⁰ and alanylglycine polymers.¹⁰¹

1.4.4 Hybrid Polymers

Hybridization of genetically engineered polymers with chemically synthesized polymers is a novel technique used to produce environmentally sensitive hydrogels.^{102–104} These hybrids take advantage of the biocompatibility of well-established synthetic polymers and structure properties of well-defined protein motifs. For example coiled-coils derived from the protein kinesin have been used to cross-link water-soluble HPMA copolymers, resulting in two-component temperature-sensitive hybrid hydrogels.¹⁰³ Halstenberg et al. have used genetic engineering and photopolymerization techniques to synthesize multifunctional protein-*graft*-poly(ethylene glycol) polymers for tissue repair.¹⁰⁵ A chemically derivatized, genetically engineered polymer, called Pronectin F, has been studied as a nonviral vector for gene delivery.¹⁰⁶

1.5 CHARACTERIZATION OF POLYMERS AND POLYMERIC DRUG DELIVERY SYSTEMS

1.5.1 THERMAL ANALYSIS

1.5.1.1 Differential Scanning Calorimetry

Thermal analysis techniques such as differential scanning calorimetry (DSC) or differential thermal analysis (DTA) measure the thermal properties of a material and allow calculation of the enthalpy (ΔH) or entropy (ΔS) changes when transformation such as crystal melting occurs. Figure 1.3 represents the schematic of a DSC apparatus. The sample is placed in a cell (*s*) and a reference sample in an identical cell (*r*). The cells are provided with independent heaters.



FIGURE 1.3 Schematic representation of a differential scanning calorimetry (DSC) apparatus: r and s are the reference and sample cells respectively; $W_r(T_r-T_p)$ and $W_s(T_s-T_p)$ are the power delivered to reference and sample cells respectively. T_r and T_s are the temperatures of the reference and sample cells respectively and T_p is the programmable temperature.

The temperature of each cell is measured continuously and compared with the instantaneous values of the programmable temperature $[T_p(t)]$. The heaters are set such that the powers delivered to the sample and reference cells are a function of the departure from the program temperature and the differential power requirement can be plotted as a function of the programmable temperature (T_p) , reference cell temperature (T_r) or sample temperature (T_s) . The enthalpy changes of a sample is determined by plotting specific heat capacity of the sample $(C_{p,s})$ vs. temperature curves obtained from the following equation:

$$C_{p,s} \frac{(Q_2 - Q_1)m_c C_{p,c}}{(Q_3 - Q_1)m_s} \tag{1.1}$$

where m_s is the mass of the sample, m_c is the mass of a standard calibrant such as alumina, $C_{p,c}$ is the specific heat capacity of a calibrant, and Q_1 , Q_2 , and Q_3 are the measured heat flows when both cells are empty, when the sample is in position, and when replaced by a calibrant, respectively.

In normal DSC measurements it is assumed that the heat capacity is thermodynamically reversible. However, for a number of processes such as glass transition temperature measurements, it can be irreversible. This results in different DSC traces of the same sample obtained by subsequent cooling and reheating. In such cases a modulated DSC (MDSC) technique is used that can separate reversible and irreversible components of the total measured heat capacity.

One important property of amorphous polymers that is measured using the DSC technique is the glass transition temperature (T_g) or the temperature of transition of a polymer from the glassy state to the rubbery phase. DSC can also be used to measure polymorphism. For example, polytetrafluoroethylene (PTFE) undergoes well-defined transitions between the crystal forms at about 35°C. In this case a number of characteristic melting signals are obtained. Melting peaks of the phase-separated homopolymers are usually identified using DSC. Polymorphism of drugs have also been extensively studied by this technique.^{107,108}

1.5.1.2 THERMOGRAVIMETRIC ANALYSIS (TGA)

Other thermal methods of analysis include thermogravimetric analysis (TGA), also known as thermogravimetry (TG). This technique measures the change in weight of a polymer as a function of temperature or time. The sample in nitrogen or in an oxidizing atmosphere is brought from ambient to a constant high temperature and the mass of the sample is recorded continuously as temperature is increased at a constant rate. At high temperature, thermal degradation of a polymer results in formation of volatile products and resulting weight loss which is measured. The usual operating temperature range is ambient to 1000°C. The TGA methods can determine loss of water or solvent, pyrolysis products, oxidative and thermal stability of polymers, and

polymeric drug delivery systems. For example, TGA has been used to determine hydration products following the enzymatic degradation of starchbased thermoplastic compounds used in prostheses.¹⁰⁹ In another example, the rheological properties of chitosan-thioglycolic acid conjugates modified with different amounts of thiol groups immobilized on the polymer have been studied by TGA to develop new scaffold materials for tissue engineering.¹¹⁰

1.5.2 DETERMINATION OF MOLECULAR WEIGHT AND MOLECULAR WEIGHT DISTRIBUTION

Unlike proteins, most synthetic polymers consist of an assembly of molecules having a distribution of molecular sizes depending on their degrees of polymerization. For such polymers the common practice is to define the molecular size in terms of average molecular mass and the mass distribution or polydispersity. Averaging is done on the basis of the number of molecules (N_i) of a particular molecular mass (M_i) . The most commonly used molecular mass averages are number average (M_n) , weight average (M_w) , z-average (M_z) , and viscosity average (M_v) . The terms are defined as follows:

$$M_n = \frac{\sum N_i M_i}{\sum N_i} \tag{1.2}$$

$$M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i} \tag{1.3}$$

$$M_z = \frac{\sum N_i M_i^3}{\sum N_i M_i^2} \tag{1.4}$$

$$M_{\nu} = \left[\frac{\sum N_i M_i^{1+\alpha}}{\sum N_i M_i}\right]^{1/\alpha}$$
(1.5)

The various instrumental methods of polymer mass measurements can be categorized into absolute and relative methods. Absolute methods include colligative property measurements such as osmometry, to determine M_n and light scattering techniques to determine M_w . Relative methods which mostly require calibration with known molecular mass standards, include gel permeation, and solution viscosity measurements.

1.5.2.1 Osmometry

The basic principle of osmometric measurements is that the chemical potential of a solvent in solution is lower than the pure solvent in a proportion equivalent to the concentration of the solute. A typical instrument used is the membrane osmometer (Figure 1.4). It consists of a measuring cell divided into two compartments by a semipermeable membrane, usually made of regenerated cellulose. Sample polymer solutions of known concentration



FIGURE 1.4 Schematic diagram of a membrane osmometer. In an enclosed system a polymer solution is separated from pure solvent by a semipermeable membrane that only allows passage of the solvent. The symbol π represents the osmotic pressure resulting from the concentration gradient.

(0.2-2%) are successively injected in the upper half of the cell. Diffusion of solvent through the membrane results in a negative pressure on the solvent side and deflects a steel membrane in the lower half of the cell. This deflection is thus proportional to the osmotic pressure and is detected as a change of capacitance of an electronic circuit. The M_n is determined using the virial equation:

$$\frac{\pi}{c} = \left(\frac{RT}{M_n} + A_2c + A_3c^2 + \cdots\right) \tag{1.6}$$

where π is the osmotic pressure, *c* is the polymer concentration, *R* is the gas constant, *T* is the temperature, A_2 and A_3 are virial constants, and M_n is the number average molecular weight. A plot of π/c vs. *c* yields a linear plot at low polymer concentrations and the intercept equals RT/M_n .

The accuracy of the method is dependent on the linearity of the curve and thus also depends on the magnitude of deviations from ideal solute behavior as indicated by the virial coefficients of second and higher order. For most systems only the second virial coefficient is important to define specific polymer-solvent interactions, whereas the higher order coefficients are negligibly small. Membrane osmometry can determine absolute molecular masses up to one million. However, the method is somewhat limited for low molar mass molecules due to the possibility of diffusion through the membrane. The limitation can be overcome by using vapor phase osmometry where lowering of vapor pressure is caused by preferential condensation of solvent from the vapor phase by a dilute polymer solution. The temperature difference involved in this process (ΔT) is usually measured to give an estimation of the M_n .

1.5.2.2 Dynamic Light Scattering

When a parallel beam of light passes through a solution, light is scattered elastically (Rayleigh scattering), due to difference in the density of the solution compared to air. Additional scattering occurs in a polymer solution as a function of its concentration as well as mass and size. The scattering of light by polymers is expressed by the Debye equation:

$$H\frac{c}{\tau} = K\frac{c}{R_{\theta}} = \frac{1}{M_w P(\theta)} + 2A_2c \tag{1.7}$$

where

$$H = \frac{32\pi^2 n^2 (\mathrm{d}n/\mathrm{d}c)^2}{3N_0\lambda^4}$$
(1.8)

In these equations τ is the turbidity (from $I = I_0 e^{-\tau l}$, Beer Lambert law), *l* is the path length, *n* is the refractive index, dn/dc is the concentration dependence of refractive index of a polymer solution, N_0 is Avogadro's number, λ is the wavelength of light in air, and $P(\theta)$ is the particle scattering factor. The dn/dc is obtained by measuring the refractive indices of a series of solutions of known concentrations. The scattered light intensity is measured at different angles to the incident beam for the same concentration range. For small molecules, a plot of $K(c/R_{\theta})$ vs. *c* is linear with intercept equal to $1/M_{W}$.

For larger molecular size it is more complex and involves the use of the Zimm plot and the following equation:

$$\frac{K_c}{R_{\theta}} = \frac{1}{M_w} + \frac{1}{M_w} \left(\frac{16\pi^2}{3\lambda_s^2}\right) \sin^2\left(\frac{\theta}{2}\right) s_z^2 + A_2 c \tag{1.9}$$

where s_z^2 is the z-average mean square radius of gyration and $\lambda_s (=\lambda/n)$ is the wavelength of light in solution. In a typical Zimm plot, double plots are constructed of K_c/R_θ against $\sin^2 (\theta/2) + kc$ where k is a scaling factor. Extrapolation of c = 0 and $\theta = 0$ gives $1/M_w$ as the y-axis intercept. In addition to M_w , the plot yields a value of A_2 (slope of q = 0 curve) and the radius of gyration s_z^2 (slope of c = 0 curve). These terms are useful in defining the behavior of the polymer molecules in solution. The radius of gyration determined in this method does not depend on the shape of the molecules. Recent use of laser light sources allows detection of light scattered at small angles for dilute polymer solutions. Thus Equation (1.9) can be rewritten as:

$$\frac{K_c}{R_\theta} = \frac{1}{M_w} + 2A_2c \tag{1.10}$$

where M_w can be obtained from the intercept of the graph of K_c/R_θ vs. c, by measuring R_θ for a single angle only.

1.5.2.3 Solution Viscometry

This method assumes that the viscosity of a polymer solution depends on the concentration and the average molecular size of the molecules. The experimental technique employed for determining M_v requires determination of the intrinsic viscosity for the polymer/solvent system, which is related to M_v based on the Mark–Houwink equation:

$$[\eta] = KM_{\nu}^{\alpha} \tag{1.11}$$

where K and α are empirical constants for a particular system. Since the hydrodynamic volume or the effective size of a molecule depends on the extent of interactions of the solute molecules with the solvent, solution viscosity in turn depends on the type of solvent used. Additionally for the same system, polymer chain branching will influence variation of $[\eta]$ with M_{ν} so that the empirical parameters K and α may not be constant for the same polymer type.

The intrinsic viscosity is experimentally determined by a viscometer by measuring the flow time of the solvent, i.e., the time for a constant volume of solvent/solution to flow between two marked points on the capillary of the viscometer. The specific viscosity is calculated from the equation:

$$\eta_{sp} = \frac{\eta_c - \eta_0}{\eta_0} = \frac{t_c - t_0}{t_0}$$
(1.12)

where t_c is the flow time for polymer solution and t_0 that for pure solvent, and from the Huggins equation:

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c$$
(1.13)

where k' is a constant.

By plotting η_{sp}/c vs. c and extrapolating to 0 concentration, the intrinsic viscosity is obtained. Values of the empirical constants K and α are obtained from absolute measurements and are used as references for specific polymer/ solvent systems.

1.5.2.4 Gel Permeation Chromatography

Gel permeation chromatography (GPC), also known as size-exclusion chromatography (SEC), is one of the most widely used methods for determining molecular mass average and polydispersity of polymers. In this case molecules are separated according to their hydrodynamic volume in solution when passed through a chromatographic bed of microporous gel particles. The gel material has a distribution of pore sizes ranging from 0.5 to 10^5 nm corresponding to the effective size range of polymer molecules. Separation of the molecules occurs by preferential diffusion of the different sized molecules in a polymer mixture into the pores. Small molecules can permeate most of the pores and hence take a longer elution time. Larger molecules are mostly excluded by the pores and find their way out quicker through the interspaces between the gel particles. Selection of column packing material with the appropriate distribution of pore sizes is crucial for effective separation of molecules in SEC. Since the hydrodynamic volume of a polymer molecule in solution determines its ability to permeate a particular pore size, changing the solvent type or temperature can affect retention time in sizeexclusion columns.

The different molecular mass fractions are characterized in SEC by their elution volume (V_e) , which is expressed as

$$K_d = \frac{(V_e - V_0)}{(V_t - V_0)} \tag{1.14}$$

where V_0 is the void volume, V_t is the total bed volume of the column, and K_d is a distribution coefficient, which indicates the relative ease of penetration of solute molecules into the pore structure. The different volume parameters are represented schematically in Figure 1.5.

For K=0 there is no penetration and K=1 indicates total permeation. K is thus directly associated with the molecular size but in practice V_e is the



FIGURE 1.5 Schematic representation of a typical gel permeation chromatography instrument demonstrating the principle of separation by size-exclusion. Large molecules elute earlier than smaller molecules thereby producing a distribution of molecular weights for a given polymer. The molecular weight of the polymer is estimated from a calibration curve of standards of known molecular weights.

experimentally measured parameter which is related to molecular mass. A calibration curve is typically generated by plotting log molecular weight vs. elution volume. The calibration curve is linear over a limited range of molecular size defined by the exclusion limits of the column in use (0 < K < 1). Hence it is crucial to choose polymer standards of similar molecular compositions as the sample polymer. The molecular masses of different fractions of polymers are obtained from the linear portion of the calibration curve, which are then used to determine the different molecular mass averages (Equations 1.2–1.5).

The ability of GPC to produce molecular mass distribution curves directly and to enable calculation of average molecular mass makes it a valuable technique for polymer characterization. SEC can separate and identify low molecular weight fractions such as monomers and oligomers from high molecular weight fractions and hence can be applied to separate or identify fragments.

1.5.3 NUCLEAR MAGNETIC RESONANCE

Nuclear magnetic resonance (NMR) spectroscopy is one of the most useful techniques for characterizing polymeric biomaterials. The basic principle behind NMR involves the detection of absorption or emission of radiation resulting from the interaction of an applied EM radiation with nuclear spins of the polymeric molecules when the energy levels in the latter are split by an external magnetic field. The primary prerequisite of NMR is that the material contains atoms whose nuclei contain an unpaired proton or neutron that possess nuclear spin properties. Since most polymeric biomaterials possess high concentration of ¹H they can be characterized by NMR. The early applications of ¹H resonance high resolution NMR to polymers were at 60 or 100 MHz (field strengths of 1.4 and 2.3T respectively) and yielded a great deal of information on the chain structures. Current high resolution NMR using superconducting magnets which generate higher frequencies (and hence better resolution) can detect difference in chain structure during polymerization. Some uses of NMR in elucidating polymer structure and polymerization processes are discussed below:

1.5.3.1 Determination of Stereochemical Configurations

There are two major ways in which the monomer units in a polymer may add to form the chain: these are the meso and racemic configurations (Figure 1.6). These configurational sequences have unique magnetic environments for the nuclei in the monomer units and yield different NMR absorption frequencies. For the meso configuration the two protons attached to C3 experience different extents of shielding from the X and Y substituents and hence have different chemical shifts leading to a four line spectrum. In contrast, for the racemic configuration the C3 protons have the same shielding due to X and Y substituents and result in a single line (singlet) absorption.



FIGURE 1.6 Stereochemical configurations of polymers. X and Y are different substituents, which can be arranged in different asymmetric arrangements.

The NMR technique is applicable to other disubstituted ethylenes, although when the size of the C substituent is larger than the methyl group, the spectra are more complex to analyze. For some polymers such as poly(methacrylic acid) and poly(acrylonitrile) the stereochemical structure can be investigated by converting the polymers to the methyl ester chemically since the assignment of its spectral lines has been established with greater certainty.

1.5.3.2 Two-Dimensional NMR

2D NMR is a two-dimensional representation of the NMR spectrum. Compared to conventional one-dimensional NMR, 2D spectra have the advantage that the NMR parameters such as chemical shift and spin-spin coupling are observed separately. This means that overlapping spectra can be resolved, simplifying peak assignments. The 2D spectra may be presented in different forms; for example, J-resolved spectra or correlated spectroscopy (COSY). J-resolved spectra are represented by chemical shifts along one frequency axis and dipolar coupling constants along the other frequency axis. XCOSY presents the data as chemical shifts of different nuclei along the two frequency axes. The 2D NMR technique has been applied to a number of polymer systems such as propylene copolymers. In this method the 2D representation is the line-to-line correspondence between ¹H and ¹³C splitting. The ¹H NMR spectra of polypropylene copolymers contain contributions from primary methyl (-CH₃), secondary methylene (-CH₂-), and tertiary methine (-CH) protons. Similarly, the ¹³C spectra comprise contributions from the corresponding carbon atoms. The peaks in the 2D spectra are due to correlated absorptions involving simultaneous ¹H and ¹³C splitting from the same group.

Electron paramagnetic resonance is a technique closely related to NMR but differs in that the spins involved are those of unpaired electrons.¹¹¹ Magnetic resonance imaging (MRI) uses this technique to construct an image based on spatial distribution and different relaxation times of spins within a sample. For example use of spin labels such as gadolinium and nitroxides in MRI has emerged as a valuable medical diagnostic tool to detect malignancies such as tumor localization or organ abnormalities exploiting variable water content of body and tissue fluids.^{112,113} Such techniques have found application in characterization of drug delivery systems as well. One interesting example of such application is the use of nitroxide spin probes to characterize the molecular environment of hydrogels and hydrophobic polyester matrices.^{114–116}



FIGURE 1.7 Simplified arrangement of optics in a transmission electron microscope (TEM). The electrons from a heated filament source are focused on the sample after passing through a series of condenser lenses. The beam after interacting with the sample penetrates and is focused on a fluorescent screen inside the microscope chamber.

1.5.4 MICROSCOPY

1.5.4.1 Transmission Electron Microscopy/Scanning Electron Microscopy

Transmission electron microscopy (TEM) is a highly magnified version of conventional optical microscopy. It can resolve structures with dimensions less than 0.2 nm and provides magnification up to million folds. The simplified schematic of a TEM is depicted in Figure 1.7. Briefly, a heated filament emits electrons, which are focused by lenses on the sample to provide high magnification. The lenses are powerful electromagnets, which bend the path of the electrons. Typically energies in the range of 60 to 100 kV are used and TEM is performed under vacuum to prevent collisions of electrons with air molecules. Critical requirement in successful TEM imaging is fixation or preservation of sample on a mount using agents such as formaldehyde, dehydration of the sample to overcome interference to evacuation, embedding of sample to provide support for sectioning, and finally proper staining for visualization.

Scanning electron microscopy (SEM) is an important supplement to TEM and is perhaps the most widely used method for characterizing polymeric drug delivery systems. The modern SEM has a resolution of about 3 nm and magnification from less than 30-fold to 300,000-fold. Usually electrons are emitted from a 1 to 630 kV heated filament source and focused on the sample surface. Secondary electrons with relatively low energy ranges from 0 to 50 eV are generated on the surface of the sample and are collected with a detector.

Although resolution is less than TEM, SEM is advantageous in the simplicity of sample preparation and the ease of operation. The SEM also provides threedimensional information about the polymeric microstructure.

The TEM and SEM have found a wide range of applications in characterization of polymeric drug delivery systems. These techniques have been particularly used to determine particle size distributions, surface topography and texture of microspheres.^{117–119}

1.5.4.2 Atomic Force Microscopy

Atomic force microscopy (AFM) is used mainly as a surface characterization tool. In AFM, a sharp tip attached to a cantilever is scanned across the sample surface (Figure 1.8). Changes in surface topography that are encountered as the tip scans the material's surface, change the interatomic attractive or repulsive forces between the surface and the tip. These forces are sensed by deflection of the cantilever on which the tip is mounted. Two common modes of operation are: (1) to vary the tip–surface distance to maintain constant interatomic force and (2) to maintain constant tip–surface distance with variable interatomic force. The height adjustments or changes in interatomic force are recorded and used to construct images of surface topography.



FIGURE 1.8 Schematic representation of an atomic force microscope. The microscopic probe consisting of a cantilever and a tip scans the sample (in X-Y plane). Alternatively the probe is fixed and the sample plane moves. The bending of the cantilever is obtained from the deflection (Z plane) of a laser beam focused on the backside of the cantilever and is used to measure the forces acting between the tip and the surface. The force changes required to counter such displacement are recorded and converted to a surface image.

To prevent damage as the tip is scanned across the surface, it is oscillated perpendicular to the surface at a high frequency, which minimizes lateral forces on the material.

The resolution of AFM images depends, in large part, on the size of the tip. A tip sharper than the smallest feature to be imaged will generally provide the best resolution. Under proper conditions, images showing individual atoms can be obtained. Thus a major feature of AFM is the ability to acquire three-dimensional images with angstrom- or nanometer-level resolution. Furthermore, imaging can be conducted without staining, coating, or other preparation, and under physiological conditions. Striking images of surfaces, biomolecules such as DNA or polymer–DNA complexes, can be obtained.^{120,121}

Newer developments in AFM methods enable chemical and mechanical information to be obtained. By attaching specific chemical groups to an AFM tip, the spatial arrangement of functional groups on a surface can be mapped. Also, because AFM is based on interaction between the tip and sample as well as surface topography, local mechanical properties, such as stiffness and friction can be determined.^{122,123}

1.5.5 Spectroscopy

1.5.5.1 Vibrational Spectroscopy

Vibrational spectroscopy detects the transition between energy levels in molecules resulting from stretching and bending vibrations of interatomic bonds. The vibrational frequencies are characteristic of particular functional groups in molecules and can provide detailed information on polymer structure.^{124,125} Two most commonly used techniques to detect changes in the vibrational energy of molecules are infrared (IR) spectroscopy and Raman spectroscopy.

1.5.5.2 Infrared Spectroscopy

When molecular vibrations cause changes in dipole moment of chemical bonds, the transitions between energy levels can be stimulated with an electromagnetic radiation. When the vibrating dipole is in phase with the electronic vector of the incident radiation the vibrations are enhanced and energy is transferred from the incident radiation to the molecule. For a simple diatomic molecule, the vibration frequency (v) is expressed as

$$\nu = \frac{(k/\mu)^{1/2}}{2\pi}$$
(1.15)

where k is the force constant, $\mu = m_1 m_2 / (m_1 + m_2)$, is the reduced mass, and m_1 and m_2 are the two masses. The detection of the energy absorption is called

IR spectroscopy. The energies of molecular vibrations in IR analysis correspond to wavelengths in the range of 2.5 to $25\,\mu m$.

1.5.5.3 Raman Spectroscopy

Raman spectroscopy is concerned with detection of light scattered inelastically by molecules interacting with incident radiation. Interaction with the incident radiation of frequency v_0 results in scattered light of frequency v_0 (elastic/ Rayleigh scattering) as well as v_0-v_{vib} (Stokes scatter) or $v_0 + v_{vib}$ (anti-Stokes scatter) when transition occurs from ground state to excited state or vice versa. The essential prerequisite for Raman spectroscopy is a change in the polarizability of the bond when vibration occurs.

1.5.5.4 Near Infrared Spectroscopy

A less widely used technique for polymer characterization, near infrared (NIR) arises from overtones of fundamental vibrations and hence is complementary to IR and Raman spectroscopy. Its advantage lies in relatively simple sample preparation. Absorption in NIR arises from the overtones v = 0 to 2 and are dominated by vibrations of hydrogen containing bonds.

The characterization of polymers by vibrational spectroscopy makes use of the concept that group frequencies, i.e., vibrational frequencies of particular chemical groups in molecules such as C=O and CH₃ tend to behave independently of the rest of the molecule. Thus the absorption frequencies of these groups appear in the same region of the spectra. By reference to standard correlation tables it is therefore possible in most cases to assign particular absorption bands to vibrations in the groups. In some cases it is possible to use internal calibration standards. For example, the vinyl acetate content of EVAc copolymers can be determined by measurement of relative intensities of acetate absorption at 1020 cm^{-1} and methylene absorption at 724 cm^{-1} and comparing with calibration curves for known compositions determined using other absolute methods. Kinetics of polymerization can also be estimated. For example, the reaction of isocyanates with amines or hydroxide groups to give urea or urethane can be easily followed by observation of the disappearance of the characteristic band of the isocyanate group at 1680 to 1610 cm^{-1} .

Vibration spectroscopy is usually performed using a variety of instruments such as dispersive IR spectrometers, Fourier transform (FT) spectrometers, and Raman spectrometers. In dispersive IR, the source, typically a black body (globar) source emits radiation over the whole IR region. Typical detectors include thermopiles, resistance thermometers (bolometers), Golay cells, or crystal diodes (measures in the microwave region). An attenuator is usually used in the path of the reference beam to equalize the sample and reference beam intensities. The movement of the attenuator is proportional to the sample absorption and displays as percentage transmission as a function of wavelength or wave number. FTIR spectrometers are used to investigate the far and near infrared region of the spectrum. Here the light source is a high intensity mercury lamp, which emits a continuous spectrum. The system contains two mirrors (one fixed and one movable) and a beam splitter. The transmitted and reflected beams emerging from the beam splitter are incident normal to the two mirrors and following reflection is recombined at the beam splitter where they produce interference effects. The beam is then passed through the sample on to the detector. The movement of the mirror causes relative phase displacement resulting in an oscillatory pattern or interferogram, which is a representation of the spectral distribution of the absorption signal reaching the detector. The movement of the mirror determines the resolution of the spectra in the different IR frequency ranges. In Raman spectrometers, because of the very low intensities of the inelastic Raman scattering, a high intensity monochromatic laser light source is used. The laser light is focused very accurately on to the sample with typical spot sizes in microns. The scattered light is observed at 90° to the incident beam using high-resolution monochromator and photomultiplier tubes.

Infrared and Raman spectroscopy are widely used in both qualitative and quantitative analysis of polymers. One application is in identification of polymers and additives. For example comparison of IR spectra from hot pressed films of plasticized poly(vinyl chloride) (PVC) before and after extraction with acetone shows that the differences arise due to the extraction of the plasticizer.¹²⁶ Identification of an unknown polymer is done by detection of characteristic absorption bands due to particular chemical groups in the polymer structure, which is then referenced to group frequency correlation tables. The IR/FTIR in conjunction with GPC has been used to determine degree of short chain branching in polymers such as polyethylene. The information can be obtained by measuring the ratio of methyl to methylene absorptions at 2965 and 2928 cm^{-1} using FTIR and comparing with the mass distribution obtained by GPC measurements.¹²⁶ Infrared also permits the detailed structural analysis of polymers based on spectral shifts arising from inter- and intramolecular interactions of chemical groups.¹²⁷ Such interactions affect both peak intensity and position.

Raman spectroscopy is a complementary technique to IR and offers advantages in that there are fewer constraints on sample form and size. It can be used for aqueous samples unlike IR and permits detection of vibrations inactive in the IR range.¹²⁸ On the other hand some IR active vibrations are inactive in Raman and these differences provide important confirmatory evidence when making assignments of particular absorptions.

1.5.5.5 MASS SPECTROSCOPY

Mass spectrometry (MS) is a highly sensitive method of characterization of polymers permitting the molecular weight determination with high resolution of detection using very small quantities of material (femtomole or less). The primary obstacle in analysis of macromolecules by MS is their low vapor pressure resulting in poor ionization in the gas phase. However recent techniques such as pyrolysis-GCMS,¹²⁹ desorption chemical ionization,¹³⁰ laser

desorption,¹³¹ and secondary ion MS (SIMS)¹³² have overcome such problems and enabled detection of compounds of molecular weight up to 10⁴ Da. Of the different MS techniques in application in recent times, two approaches have gathered most interest, namely electrospray ionization (ES) and matrix-assisted laser desorption-time of flight (MALDI-TOF) mass spectrometry.

1.5.5.6 Electrospray Mass Spectrometry

In electrospray (ES), macromolecules are detected as multicharged ions; the number of cations (ES+) and anions (ES-) bound to each molecule being greater, the higher the molecular mass. Biomolecules such as proteins, biopolymers, and nucleic acids can have many functional groups in their backbone that are relatively easily ionized. Figure 1.9 shows the scheme of a typical ES/MS system. In this system a diluted sample solution (usually in acetone, acetonitrile, methanol and water) is injected at a slow flow rate (microliters to nanoliters per minute), using a needle housed in a cylindrical electrode kept at a high potential (gradients in the order of 3 kV/cm). The electrical field (E) generated at the needlepoint disperses the flow as electrically charged droplets. Solvent evaporation occurs in the zone between the needle and capillary under a flow of dry nitrogen (at a temperature of 50-100°C, flow of 100 cm³/sec and pressure of 760 torr) and individual positively or negatively charged macro ions are formed according to the polarity of the field. To determine the molecular weight of the ions, the ES is connected to a suitable MS. The interface is composed of a capillary and a skimmer kept under vacuum $(10^{-3}-10^{-4} \text{ torr})$ by a first pumping stage. Under the influence of an electric field generated by electrostatic lenses, the ions coming out of the capillary are transported to the skimmer by supersonic jet expansions and enter



FIGURE 1.9 Schematic diagram of a typical electrospray mass spectrometry (ES/MS) system. The sample is injected through a needle housed in a cylindrical electrode kept at a high potential. The electric field generated at the needle point converts the sample to electrically charged droplets which evaporate in the capillary and the sample ions are collected and analyzed by a quadrupole.

the MS, which is generally a quadrupole. Although the range of mass analyzable by these instruments is not very high (up to 4000 Da), high molecular weight polymers are also analyzable because the macroions formed are usually multicharged. Thus the MS spectrum of BSA for example having a mass of about 66,500 Da, shows a series of peaks with a maximum at about m/z = 1622 (corresponding to the molecule having 41 positive charges).

The use of ES/MS for characterization of synthetic polymers is usually directed towards polar compounds, which are more easily ionized. Thus MS has been successfully applied for analyzing water-soluble PEGs up to 5 MDa and poly(amidoamine) (PAMAM) dendrimers of up to generation 10 (1 MDa).¹³³ However, apolar polymers like polystyrene¹³⁴ have also recently been successfully analyzed. In case of natural polymers (such as polysaccharides), usually ES/MS is performed after controlled degradation to oligomers. If suitably purified and by adjusting the potential of the skimmer,¹³⁵ it is possible to identify the different types of fragmentation and also analyze how single saccharide units are connected to each other.

The ES/MS is used to analyze the structure of peptides and proteins by determining the sequence of amino acid units, identifying protein metabolites, position of disulfide bridges, identifying covalently bound ligands and characterizing active enzyme sites.¹³⁶ These are achieved by peptide mapping/MS, which consists of analyzing the peptides obtained by protein fragmentation using MS and recording their spectra in a library.¹³⁶ ES/MS has also found application in nucleic acid sequencing¹³⁷ of various oligonucleotides, and identifying noncovalent associations in DNA-protein complexes.¹³⁸ MS interfaced with liquid chromatography (LC) (LCMS) is another widely used tool which enables analysis of macromolecules using a wide range of flow (from nanoliter to milliliter per minute).¹³⁹ Using ultrasonic nebulizers it is possible to achieve a suitable spray into the MS regardless of the solvent characteristics. Also LCMS can be interfaced with capillary electrophoresis (CE), which separates complex biological mixtures due to different charge/ mass ratios of its components. The use of this method has found application in separation and identification of peptides and proteins including synthetic peptides,¹⁴⁰ glycoproteins,¹⁴¹ oligonucleotides,¹⁴² and oligosaccharides.¹⁴³ In the field of synthetic oligomers and polymer analysis, GPC is a powerful tool to determine molecular weight and polydispersity index. Mass spectrometry can be interfaced with GPC to analyze polymers.¹⁴⁴ In this way elution times of each species is associated with the corresponding molecular mass rather than the relative calibration standard curve that is commonly used in GPC.

1.5.5.7 MALDI-TOF Mass Spectrometry

MALDI-TOF is a soft ionization technique in which the sample mixed with a specially selected solid matrix, is irradiated with a laser light. The matrix present in large molar excess to the sample ($\sim 1:2000$) is chosen for its ability to absorb the laser light and protect the analyte, transferring energy to it in a way that allows desorption and ionization of molecules without significant

fragmentation. For mass separation of ions in MALDI it is usually combined with a TOF MS which offers high extraction efficiency, high transmission, and high mass detection capability.¹⁴⁵

In a MALDI-TOF spectrometer a pulsed laser such as Nd-YAG (355 and 266 nm), N_2 (337 nm) or TEA-CO₂ (10.6 µm) with pulse widths in the 1 to 100 ns range produces a simultaneous desorption in discrete packets of a large number of singly charged molecular ions (multiple charged ions are usually not observed), as proton or alkaline metal (Li, Na, or K) ion adducts. The ions are accelerated to a fixed kinetic energy by an electric potential applied just above the sample surface and the velocity of each ion will be proportional to its mass/ charge ratio. By passing through a field free drift tube, ion species are separated into a series of ion packets each traveling with a velocity characteristic of its mass. A detector is present at the end of the flight tube which obtains the spectrum as a function of time.

MALDI-TOF is advantageous compared to ES/MS due to the absence of substantial fragmentation, high sensitivity (femtomolar), unlimited mass range (>1.5MDa), easy sample handling, and short analysis time. MALDI-TOF is used in a wide number of applications including structural characterization of polymers, ^{131,146} proteins, ^{147,148} oligosaccharides, ¹⁴⁹ and oligonucleotides. ¹⁵⁰ In determination of molecular weight distribution of polymers with large polydispersity, MALDI-TOF tends to underestimate the higher masses due to lower abundance, which overlaps with noise levels. This can be overcome by interfacing GPC with MALDI-TOF where the eluted fractions from GPC are analyzed offline by MALDI.

1.5.5.8 X-ray Photoelectron Spectroscopy

The principle of operation of X-ray photoelectron spectroscopy (XPS) is that when the surface of a material is bombarded with an x-ray, electrons are emitted and the energy of the emission is detected to measure the binding energy. The exciting radiation is a monochromatic beam of soft x-rays. The energy of emission of the electrons is low and hence most of the emitted electrons are recaptured. Only those electrons that are very close to the surface can escape and are detected. The energies of the emitted electrons are typically characteristic of the binding states of the substrate surface atoms. The bombardment process with an x-ray can cause three different phenomena. Excitation may cause direct ejection of a core electron from an atom (photoionization). This may cause reorganization of the valence electrons and subsequent emission of electrons from the valence band causing ionization (shake-off). The valence electron could also move to a higher unoccupied energy level (shake-up). Typically the kinetic energies of the electrons emitted by these phenomena are in the order: $E_{\text{shake-off}} < E_{\text{shake-up}} < E_{\text{photoionization}}$. Analysis of surface energy is performed in a XPS spectrophotometer (Figure 1.10). Here samples are solution cast or melt processed as very thin films and bombarded with x-rays. The emitted electrons are passed through a magnetic or electrostatic field under high vacuum ($\sim 10^{-8}$ torr) causing them to



FIGURE 1.10 Schematic diagram of an x-ray photoelectron spectroscopy (XPS) instrument. A sample is bombarded with x-rays and the emitted electrons are passed through a magnetic field causing them to travel in a curved path to the detector. The binding energy of the ejected electrons is representative of its surface characteristics.

travel on a curved path with the curvature determined by the velocity of the electron. The binding energy (E_b) of a core electron is related to its measured kinetic energy by:

$$E_k = h\nu - E_b - \Phi \tag{1.16}$$

where E_k is the measured kinetic energy of the electron, hv is the energy of the exciting radiation, and Φ is the work function whose value depends on the sample and the spectrometer. The above theory assumes that the sample is neutral. However, upon bombardment with x-rays, the sample may become charged which may affect the work function. Charge effects are usually reduced by electrically grounding the sample. In practice the spectral peaks are usually referenced to standard values and accordingly corrected for energy shifts due to partial ionization of samples.

Referencing is usually done by depositing a thin coating of a suitable standard material that will provide a signal corresponding to a precisely known binding energy within the range of interest. For example C1s from a hydrocarbon has a value of 285 eV and 4f of gold has a value of 84 eV. Sometimes the correction is only a fraction of 1 eV and can be ignored. Alternatively C1s is used as an internal standard being present in most materials.

There are a large number of applications of XPS for polymer characterization in the literature. One mechanism that is studied extensively is oxidation effects.¹⁵² Degradative phenomenon associated with oxidation, such as UV promoted photo-oxidation, primarily take place near the surface of polymers causing reduction of its mechanical properties. Normally the O 1*s* and the C 1*s* spectra are examined. For example the O 1*s* spectrum has a peak corresponding to the binding energy of about 533 eV with a shoulder at 535 eV. Upon desiccation in the presence of P₂O₅, this shoulder usually disappears and is thus attributed to absorbed water. The 533 eV peak is attributed to C=O and shows a small shoulder on the high binding energy side of the main C 1*s* peak in polypropylene. XPS has found application in distinguishing different carbon containing species on the surface of polymeric biomaterials.¹⁵³ XPS is also used to characterize the extent of protein adsorption or binding on a variety of different biosensor interfaces which are modified with polymers in order to inhibit nonspecific adsorption from concentrated protein solutions.¹⁵⁴

1.5.6 GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis (PAGE) is a highly versatile technique primarily applied to determine protein molecular weight. It involves migration of charged molecules through a static medium under the action of an electric field. Proteins are usually denatured to a constant conformation by cleavage of disulfide bonds using denaturants like dithiothreitol and by adding detergents (sodium dodecyl sulfate, SDS) to bind to proteins at a constant level per amino acid residue. The resulting aggregates have a net charge, which is proportional to their molecular weight M. The terminal velocity of the aggregates V_0 is given by,

$$V_0 = \frac{K_1 E}{\varepsilon M^x} - K_2 E \tag{1.17}$$

where E is the magnitude of the field (volts per meter), ε is the relative permittivity of the gel bed in which the aggregates are moving, K_1 and K_2 are constants of proportionality, and 0 < x < 1, where x is related to the characteristic length dimension of the aggregate. Thus in SDS-PAGE progress of larger (heavier) proteins is retarded relative to the smaller (lighter) ones. The gel most commonly used is a copolymer of acrylamide and N.N'-methylene-bisacrylamide. The relative concentrations of these comonomers determine the mechanical properties of the gel. The range of molecular weights that can be resolved on a gel is also determined in part by the gel concentration. For SDS PAGE, gel preparation also involves appropriate buffering of the aqueous medium. A large number of different gel and buffer systems exist depending on the types of protein or other macromolecules to be characterized.¹⁵⁵ The composition and pH of the buffer in SDS PAGE determines whether a protein retains its native undissociated state or whether it denatures and dissociates into individual polypeptide subunits. By running native and denatured proteins on separate gels it is possible to determine how many subunits there are in the protein. In the case of native gels the charge on individual proteins is sensitive to buffer pH, providing additional means of separation and characterization. Gel electrophoresis is a relative method and hence internal calibration standards (molecular markers) are run together with the sample of interest. Molecular markers are chosen to have SDS binding characteristics like the sample molecules in the chosen buffer. Gel electrophoresis is widely used for determining the variety, molecular masses and relative quantities of constituent polypeptides in protein samples.¹⁵⁵ The method is also applied to glycoproteins,¹⁵⁶ nucleic acids (DNA and RNA),¹⁵⁷ polysaccharides,¹⁵⁸ and some lipids and glycolipids.¹⁵⁹

For further details on characterization of polymers the reader is referred to the *Comprehensive Desk Reference of Polymer Characterization and Analysis*.¹⁶⁰

1.6 CONCLUDING REMARKS

Design of a particular polymeric delivery system for proteins and nucleic acids will require a thorough understanding of the physicochemical properties of a given polymer. As discussed in this chapter, several techniques can be used for characterization of polymers and polymeric drug delivery systems in terms of chemical structures, molecular weight, particle size, surface morphology, and phase transition temperature.

1.7 ABBREVIATIONS

^{99m} Tc	technetium-99m
A (Ala)	alanine
ADA	adenosine deaminase
AFM	atomic force microscopy
BPA	bisphenol-A
CE	capillary electrophoresis
COSY	correlated spectroscopy
DNA	deoxyribonucleic acid
DSC	differential scanning calorimetry
DTA	differential thermal analysis
ELP	elastin-like polymer
EM	electromagnetic
EPR	enhanced permeability and retention
ES	electrospray
EVAc	poly[ethylene-co-(vinyl acetate)]
F	phenylalanine
FDA	Food and Drug Administration
FT	Fourier transform
G (Gly)	glycine
GC	gas chromatography
GPC	gel permeation chromatography
HPMA	N-(2-hydroxypropyl)methacrylamide
HPMC	hydroxypropyl methylcellulose
HSA	human serum albumin
Нур	hydroxyproline
Ι	isoleucine
IR	infrared
kDa	kilodaltons
LC	liquid chromatography
MALDI-TOF	matrix-assisted laser desorption-time of flight
MDSC	modulated differential scanning calorimetry
M_n	number average molecular weight

MRI	magnetic resonance imaging
MS	mass spectrometry
M_w	weight average molecular weight
M_z	z-average molecular weight
NIR	near infrared
NMR	nuclear magnetic resonance
P (Pro)	proline
PAGE	polyacrylamide gel electrophoresis
PAMAM	poly(amidoamine)
PEG	poly(ethylene glycol)
PEO	poly(ethylene oxide)
PGA	poly(glycolic acid)
PHEA	β -poly(2-hydroxyethyl aspartamide)
PHEG	poly[N-(2-hydroxyethyl)-L-glutamine]
PLA	poly(lactic acid)
PLGA	poly(D, L-lactide-co-glycolide)
PMMA	poly(methyl methacrylate)
PPE	poly(phosphoester)
PTFE	polytetrafluoroethylene
PVC	poly(vinyl chloride)
rhBMPs	recombinant human bone morphogenetic proteins
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SEC	size-exclusion chromatography
SELP	silk elastin-like protein polymer
SEM	scanning electron microscopy
SIMS	secondary ion mass spectrometry
SLP	silk-like polymer
TEM	transmission electron microscopy
TG	thermogravimetry
TGA	thermogravimetric analysis
UV	ultraviolet
V	valine
XPS	x-ray photoelectron spectroscopy

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2 Step-Growth and Ring-Opening Polymerization

Rajendra P. Pawar and Abraham J. Domb

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2.1 INTRODUCTION

Condensation polymerizations are those which yield polymers with repeating units having lesser atoms than present in the monomers from which they are formed. This type of polymerization involves the elimination of a small molecule (e.g., H_2O , HCl). Addition polymerizations are those which yield polymers with repeat units having identical molecular formula of those of the monomers from which they are formed, as well as losing the unsaturation such as double bonds that transform to connections between the monomer units. This type of polymerization does not eliminate any small molecule and the entire monomer molecule becomes part of the polymer.¹

The polymerization processes in which a polymer chain grows step-wise by reactions that can occur between any two molecular species are known as *step-growth polymerizations*. In step-growth polymerization the degree of polymerization increases steadily throughout the reaction. Step-growth polymerization proceeds by the reaction of two different functional groups. The monomer disappears rapidly while the molecular weight builds up slowly. The polymerization processes in which a polymer chain grows only by reaction of a monomer with a reactive end-group on the growing chain are known as *chain-growth polymerizations*. In chain-growth polymerization high degrees of polymerization are attained at low monomer conversion. The chain-growth polymerization requires an initiator and molecular weight builds up rapidly.

Ring-opening polymerization shares the features of condensation and addition polymerization mechanisms. Ring-opening polymerization takes place by condensation polymerization and proceeds without the splitting of a small molecule. The driving force for ring-opening polymerization is also derived from the loss of the unsaturation ring. Ring-opening polymerization takes place only in cyclic compounds and possesses at least one hetero atom. Ring-opening polymerization also involves an initial ring-opening of the cyclic monomer followed by polyaddition. The resulting polymer is normally linear (Scheme 2.1).



SCHEME 2.1



SCHEME 2.2



SCHEME 2.3

2.2 STEP-GROWTH POLYMERIZATION

Step-growth polymerization is the process in which the polymer chain grows stepwise to form a polymer by the reaction between two bifunctional molecular species such as diacids and diamines or from a bifunctional molecule such as hydroxyacetic acid (Scheme 2.2).

Linear step-growth polymerization requires that the monomers will react at a 1:1 stoichiometry of mutually reactive functional groups. The step-growth polymerization process must be carefully designed to avoid side reactions which may result in the loss of monomer functionality or polymer degradation. It is essential to keep the stoichiometry between the two types of monomers throughout the polymerization if a high molecular weight polymer is desired. Another issue is the possibility of depolymerization through an inter- and intra-bond interchange resulting in a low molecular weight polymer or even conversion into oligomers. For example poly(lactic acid) upon overheating converts into lactide (Scheme 2.3).

The introduction of branching networks is accomplished using trifunctional monomers. Many step-growth polymers are attained through the combination of controlled end group functionality, extent of branching, and the molecular weight.¹

2.2.1 MECHANISM AND KINETICS

The condensation polymerization takes place by as addition–elimination type of reaction. In this reaction, the substrate is attacked by a nucleophile to form a carbanion intermediate from which the leaving group is eliminated with formation of a polymer (Scheme 2.4).²

The assumption of equal reactivity of functional groups simplifies the kinetics of step-growth polymerization since a single rate constant applies to each of the step-wise reactions.¹ It is usual to define the overall rate of reaction



SCHEME 2.4

as the rate of decrease in the concentration of one or other of the functional groups, i.e., in general terms for equimolar stoichiometry:

Rate of reaction
$$= -\frac{d[A]}{dt} = -\frac{d[B]}{dt}$$
 (2.1)

Most of the step-growth polymerization involves bimolecular (A + B) reactions which are often catalysed. Thus neglecting elimination products in polycondensations, the general reaction is:

 $\sim \sim \sim \sim A + B \sim \sim \sim + Catalyst \longrightarrow \sim \sim \sim AB \sim \sim \sim + Catalyst$

and so the rate of reaction is given by:

$$-\frac{\mathrm{d}[A]}{\mathrm{d}t} = k'[A][B][\mathrm{Catalyst}]$$
(2.2)

where k' is the rate constant for the reaction. Since the concentration of a true catalyst does not change as the reaction proceeds, it is usual to simplify the expression by letting k = k' [Catalyst] giving:

$$-\frac{\mathrm{d}[A]}{\mathrm{d}t} = k[A][B] \tag{2.3}$$

for equimolar stoichiometry [A] = [B] = c and Equation (2.3) becomes:

$$-\frac{\mathrm{d}c}{\mathrm{d}t} = kc^2 \tag{2.4}$$

This equation may be integrated by letting $c = c_0$ at t=0:

$$\int_{c_0}^{c} -\frac{\mathrm{d}c}{c^2} = \int_0^t k \,\mathrm{d}t \tag{2.5}$$

and gives

$$\frac{1}{c} - \frac{1}{c_0} = kt$$
 (2.6)

which may be written in terms of the extent of reaction (p) that is given by:

$$p = \frac{(N_0 - N)}{N_0} \tag{2.7}$$

where N_0 is the initial number of molecules and N is the number of molecules presented after time t in the polymerization reaction, by recognizing that $c_0/c = N_0/N$ and applying Equation (2.7):

$$\frac{1}{(1-p)} - 1 = c_0 kt \tag{2.8}$$

This equation also applies to reactions which proceed in the absence of a catalyst, though the rate constant is different and obviously does not include a term in catalyst concentration.

Certain step polymerizations are self-catalyzed, i.e., one of the types of functional group also acts as a catalyst (e.g., carboxylic acid groups in a polyesterification). In the absence of an added catalyst, the rate of reaction for such polymerization is given by:

$$-\frac{d[A]}{dt} = k''[A][B][A]$$
(2.9)

assuming that the A groups catalyze the reaction. Again letting [A] = [B] = c, Equation (2.9) becomes:

$$-\frac{\mathrm{d}c}{\mathrm{d}t} = k''c^3 \tag{2.10}$$

which upon integration as before gives:

$$\frac{1}{c^2} - \frac{1}{c_0^2} = 2k''t \tag{2.11}$$

or in terms of the extent of reaction:

$$\frac{1}{\left(1-p\right)^2} - 1 = 2c_0^2 k'' t \tag{2.12}$$

Equations (2.8) and (2.12) have been derived assuming that the reverse reaction (i.e., depolymerization) is negligible. This is satisfactory for many polyadditions, but for reversible polycondensations, the elimination product needs to be removed continuously as it is formed.

2.2.2 POLYMERS SYNTHESIZED BY STEP-GROWTH POLYMERIZATION

2.2.2.1 Polyesters

Polyesters are synthesized by the condensation polymerization of dicarboxylic acids and diols, or hydroxy alkyl carboxylic acids.³ Examples for both polymerizations are (Scheme 2.5):



SCHEME 2.5

The ω -hydroxycaproic acid on condensation gave polycaprolactone. At every condensation reaction the polymer chain grows but remains a ω -hydroxy carboxylic acid, hence it further reacts to form a longer polymer chain.

Dimethyl terephthalate and ethylene glycol at a 1:1 mole ratio heated at 200°C in the presence of calcium acetate form oligomers which at 300°C gave poly(ethylene terephthalate) and methanol as byproducts. This polymer is used in synthetic fibers, films bottles, and for vascular grafts.

2.2.2.2 Poly(ortho esters)

Poly(ortho ester) can be synthesized by the reaction of ketene acetal and an alcohol, to form the orthoester bond (-O-C-OO-) between the polymer units (Scheme 2.6). For example, 3,9-bis(methylene) 2,4,8,10-tetraoxaspiro[5,5] undecane on condensation with 1,6-hexanediol forms a poly(ortho ester) (Scheme 2.6). The reaction proceeds at room temperature using tetrahydro-furan as solvent and acid catalyst. The preparation of poly(ortho esters) from diol and diketene acetals is similar to the preparation of polyurethanes from diol and diisocynate. Both reactions proceed without the formation of a byproduct. Poly(ortho esters) have been tested as carriers of drugs like insulin and antibiotics.⁴

2.2.2.3 Polyamides

Adipic acid and hexamethylene diamine salts are prepared first and then the salts are heated at higher temperature to form Nylon 6.6, a polyamide.⁵



 $\begin{bmatrix} 0 & 0 \\ -0 - C - (CH_2)_4 - C - 0^- \end{bmatrix} \begin{bmatrix} + \\ H_3N - (CH_2)_4 - NH_3 \end{bmatrix}$

(b) Preparation of Nylon 6



heat

SCHEME 2.7

The polymerization reaction takes place at a higher temperature than the melting points of the reactants and the polymer (Scheme 2.7).

2.2.2.4 Polyimides

The condensation reaction between cyclic anhydrides and diamines afforded polyimides. The reaction takes place in two steps, the first step involves condensation of the cyclic anhydrides and diamines in an anhydrous solvent like dimethylacetamide to form a polyamic acid intermediate followed by

dehydration at elevated temperature (Scheme 2.8). These polymers have high thermo-oxidative stability and are good electrical insulators.⁵

2.2.2.5 Polyethers

Polyethers can be prepared by step-growth condensation from the reaction of oxygen gas and 2,6-dimethyl phenol in the presence of cuprous chloride and pyridine (Scheme 2.9). Due to the high impact strength and resistance to mineral and organic acids this polymer is used for the manufacturing of machine parts and containers.⁵

Similarly, poly(ethylene glycol) can be synthesized from the reaction of ethylene glycol and dichloroethane (Scheme 2.10).



SCHEME 2.8



SCHEME 2.9

 $HO - CH_2 - CH_2 - OH + CI - CH_2 - CH_2 - CI \xrightarrow{CuCl_2} (-CH_2 - CH_2 - O) + 2n HCI$

SCHEME 2.10



SCHEME 2.11

2.2.2.6 Polysulfones

Polysulfones are synthesized by the nucleophilic substitution of alkali salts of biphenates with activated aromatic dihalides.⁵ The disodium salt of bisphenol on reaction with dichlorodiphenyl sulfone forms a polysulfone (Scheme 2.11) (Table 2.1).

2.3 CHAIN-GROWTH POLYMERIZATION

Chain-growth polymerization is an important method of polymer preparation which involves the addition of unsaturated molecules to a rapidly growing chain. The unsaturated compounds undergo the chain-growth polymerization (Scheme 2.12).

The growing polymer in chain-growth polymerization is a free radical and proceeds through chain mechanism. Chain-growth polymerization is induced by the addition of free radical-forming reagents or by ionic initiators. It involves three steps: initiation, propagation, and termination.⁵

2.3.1 FREE-RADICAL CHAIN GROWTH POLYMERIZATION

The free-radical chain growth polymerization takes place in three steps.

Initiation: This step involves formation of the free-radical active center which takes place in two steps. The first step is the formation of free radicals from an initiator and the second step is the addition of one of these free radicals to a monomer (Scheme 2.13).



SCHEME 2.12



SCHEME 2.13

SCHEME 2.14

Propagation: This step involves growth of the polymer chain by rapid sequential addition of monomer to the active center. The time required for each monomer addition is in the order of a millisecond (Scheme 2.14).

Termination: In this step the growth of the polymer chain is terminated. The termination involves the coupling together of two growing chains to form a single polymer molecule or disproportionation to form two nonsymmetrical chains. Coupling leads to initiator fragments at both ends of the polymer chain, whereas disproportionation results in an initiator fragment at one end. The mechanism of termination involves bimolecular reaction of growing polymer chains (Scheme 2.15).⁵



2.3.1.1 Kinetics

Initiation: The initiation of polymerization reaction is a two step process involving:

1. The decomposition of the initiator into primary radicals

$$I \xrightarrow{k_d} 2R^{\bullet} \tag{2.13}$$

2. The addition of a monomer to the primary free radical

$$R^{\bullet} + M \xrightarrow{k_a} RM_i^{\bullet} \tag{2.14}$$

The constants for initiator dissociation and monomer addition are k_d and k_a , respectively. Since initiator dissociation [Equation (2.13)] is much slower than monomer addition [Equation (2.14)], the first step of the initiation is the rate limiting step. Some of the initiator radicals may undergo side reactions, such as combination with another radical, that preclude monomer addition. Therefore only a fraction, f, of the initial initiator concentration is effective in the polymerization process. Also, decomposition of each initiator molecule produces a pair of free radicals, either or both of which can initiate polymerization. Based on these observations, the rate expression for initiation may be written as:

$$R_i = \frac{\mathrm{d}[M^{\bullet}]}{\mathrm{d}t} = 2fk_d[I] \tag{2.15}$$

where [I] represents the initiator concentration.

Propagation: The successive addition of monomers during propagation may be represented as follows:

$$RM_1^{\bullet} + M \xrightarrow{k_p} RM_2^{\bullet}$$
 (2.16)

$$RM_2^{\bullet} + M \xrightarrow{k_p} RM_3^{\bullet}$$
 (2.17)

In general:

$$RM_{x}^{\bullet} + M \xrightarrow{k_{p}} RM_{x+1}^{\bullet}$$
 (2.18)

The above scheme is based on the assumption that radical reactivity is independent of chain length. Essentially, this means that all the propagation steps have the same rate constants k_p . In addition, propagation is a fast process. For example, under typical reaction conditions, a polymer of molecular weight of about 10⁷ may be produced in 0.1 s. It may therefore be assumed that the number of monomer molecules reacting in the second initiation step is insignificant compared with that consumed in the propagation step. Thus the rate of polymerization equals essentially the rate of consumption of monomers in the propagation step. The polymerization rate can therefore be written as:

$$R_p = -\frac{d[M]}{dt} = k_p[M^*][M]$$
(2.19)

where $[M^*] = \sum RM_x^{\bullet}$, i.e., the sum of the concentrations of all chain radicals of type RM_x .

Termination: Chain growth may be terminated at any point during polymerization by either or both of two mechanisms.

1. Combination (coupling):

$$M_x^{\bullet} + M_y^{\bullet} \xrightarrow{k_{tc}} M_{x+y} \tag{2.20}$$

2. Disproportionation:

$$M_x^{\bullet} + M_y^{\bullet} \xrightarrow{k_{td}} M_x + M_y$$
 (2.21)

If there is no need to distinguish between the two types of termination, which in any case are kinetically equivalent, then termination may be represented as:

$$M_x^{\bullet} + M_y^{\bullet} \xrightarrow{\kappa_t} P \tag{2.22}$$

where $k_t(k_{tc} + k_{td})$, k_{tc} and k_{td} are the rate constants for overall termination process, termination by coupling, and termination by disproportionation, respectively. The termination rate is given by:

$$R_t = \frac{-\mathrm{d}[M^{\bullet}]}{\mathrm{d}t} = 2k_t [M^{\bullet}]^2$$
(2.23)

The factor of 2 arises from the fact that, at each incidence of termination reaction, two radicals disappear.

Over the course of polymerization (at steady state), the total radical concentration remains constant. This means that radicals are being produced and destroyed at equal rates (i.e., $R_i = R_t$). It follows from Equations (2.15)

and (2.23) that

$$[M^{\bullet}] = \left(\frac{f \, k_d}{k_t}\right)^{1/2} [I]^{1/2} \tag{2.24}$$

Since the overall polymerization rate is essentially the rate of monomer consumption during propagation, substitution of Equation (2.24) into Equation (2.19) yields:

$$R_p = k_p \left(\frac{fk_d}{k_t}\right)^{1/2} [I]^{1/2} [M]$$
(2.25)

Equation (2.25) predicts that the rate of polymer formation in free radical polymerization is first order in monomer concentration and half order in initiator concentration. This assumes, of course, that the initiator efficiency is independent of monomer concentration. This confirmation of first-order kinetics with respect to the monomer concentration suggests an efficiency of utilization of primary radicals, f, near unity. Even where the kinetics indicates a decrease in f with dilution, the decreases have been invariably small. For undiluted monomers, efficiencies near unity are not possible.⁵

2.3.1.2 Polymers Synthesized by Free Radical Chain-Growth Polymerization

Ethylene polymerizes to form polyethylene when heated at 200° C in the presence of benzoyl peroxide. The polymerization steps are as described in Scheme 2.15 where X = H.

2.3.2 IONIC CHAIN-GROWTH POLYMERIZATION

Chain polymerization of unsaturated monomers can also be affected by active centers which possess ionic charges. The ionic polymerizations are of two types: the ionic polymerization in which the active center is positively charged is called *cationic polymerization*, and similarly, the ionic polymerization in which the active center is negatively charged is called *anionic polymerization*. Initiation of ionic polymerization usually involves the transfer of an ion or an electron to or from the monomer. The polymerization mechanism for each monomer is related to the polarity of the monomers and the Lewis acid-base strength of the ion formed.¹

Similar to free radical polymerization, ionic polymerization takes place in three steps: *initiation*, *propagation*, and *termination*.

2.3.2.1 Kinetics

For the purpose of establishing the kinetics of generalized cationic polymerization, A represents the catalyst and RH the cocatalyst, M the

monomer, and the catalyst–cocatalyst complex H^+AR^- . Then the individual reaction steps can be represented as follows:

$$A + RH \stackrel{k}{\longleftrightarrow} H^+ AR^- \tag{2.26}$$

$$H^+AR^- + M \xrightarrow{k_i} HM^+AR^- \tag{2.27}$$

$$HM_n^+AR^- + M \xrightarrow{k_p} HM_{n+1}^+AR^-$$
(2.28)

$$HM_n^+ AR^- \xrightarrow{k_i} M_n + H^+ AR^-$$
(2.29)

$$HM_n^+AR^- + M \xrightarrow{k_n} M_n + HM^+AR^-$$
(2.30)

The rate of initiation R_i is given by:

$$R_i = k_i [H^+ A R^-][M] = k_i K[A][RH][M]$$
(2.31)

As usual, the square brackets denote concentration. If the complex H^+AR^- is readily converted to cation and the initiation step is the rate-limiting step, then the rate of initiation is independent of the monomer concentration. Since $AR^$ remains in close vicinity of the growing center, the termination step is first order:

$$R_t = k_t [M^+] (2.32)$$

Where $[M^+]$ is the concentration of all chain carriers $[HM_n^+AR^-]$. The retention of the terminating agent AR^- in the vicinity of the chain carrier is responsible for the primary difference between the kinetics of cationic polymerization and that of free-radical polymerization. Assuming that the steady state holds, then $R_i = R_t$ and:

$$[M^{+}] = \frac{Kk_{i}}{k_{t}} [A][RH][M]$$
(2.33)

The overall rate of polymerization, R_p is given by:

$$R_p = k_p[M^+][M] = K \frac{k_i k_p}{k_i} [A][RH][M]^2$$
(2.34)

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The number-average degree of polymerization, assuming predominance of termination over chain transfer, is

$$\bar{X}_{n} = \frac{R_{p}}{R_{i}} = \frac{k_{p}}{k_{t}} \frac{[M^{+}][M]}{[M^{+}]} = \frac{k_{p}}{k_{t}} [M]$$
(2.35)

If, on the other hand, chain transfer dominates, then:

$$\frac{-}{N_n} = \frac{R_p}{R_{tr}} = \frac{k_p [M^+][M]}{k_t [M^+][M]} = \frac{k_p}{k_{tr}}$$
(2.36)

In this case, the average degree of polymerization is independent of both the concentration of the monomer and the concentration of the catalyst. Available kinetic data tend to support the above mechanism.⁵

Available kinetic data for the polymerization of methylacrylonitrile by potassium amide in liquid ammonia support the following steps in the mechanism of anionic polymerization:⁵

$$KNH_2 \stackrel{K}{\longleftrightarrow} K^+ + NH_2^-$$
 (2.37)

$$NH_2^- + M \xrightarrow{k_i} NH_2M^-$$
 (2.38)

$$NH_2M_n^- + M \xrightarrow{k_p} NH_2M_{n+1}^-$$
 (2.39)

$$NH_2M_n^- + NH_3 \xrightarrow{k_{lr}} NH_2M_nH + NH_2^-$$
 (2.40)

Considering the relatively high dielectric constant of the liquid ammonia medium, the counterion K^+ can be neglected. Assuming steady-state kinetics:

$$R_i = k_i [NH_2^-][M]$$
(2.41)

$$R_t = k_{tr}[NH_2 - M_n^-][NH_3]$$
(2.42)

Thus from the initiation step:

$$[NH_2 - M_n^-] = \frac{k_i}{k_{tr}} \frac{[NH_2^-][M]^2}{[NH_3]}$$
(2.43)

the rate of polymerization becomes:

$$R_p = k_p [NH_2 - M_n^-][M] = k_p \frac{k_i}{k_{tr}} \frac{[NH_2^-][M]^2}{[NH_3]}$$
(2.44)

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Given the predominance of transfer reactions, the degree of polymerization X_n is given by:

$$\bar{X} = \frac{R_p}{R_t} = \frac{k_p}{k_{tr}} \frac{[M]}{[NH_3]}$$
 (2.45)

2.3.2.2 Cationic Polymerization

Monomers with electron-donating groups like isobutylene form stable positive charges and are readily converted to polymers by cationic catalysts. Strong Lewis acids like boron trifluoride and aluminum chloride can initiate cationic polymerization in the presence of a cocatalyst like water, which serves as Lewis base or source of protons. During initiation a proton is added to the monomer to form a carbonium ion which forms an association with the counterion. Propagation involves the consecutive additions of monomer molecules to the carbonium ion at the growing chain end. Termination involves the rearrangement to produce a polymer with an unsaturated terminal unit. Unlike free radical polymerization, termination by combination of two cationic polymer growing chains does not occur.⁵

2.3.2.3 Anionic Polymerization

Monomers suitable for anionic polymerization generally contain electronwithdrawing groups. The monomers include styrene, acrylonitrile, butadiene, acrylates, ethylene oxide, and lactones. The initiator in anionic polymerization may be any compound providing a strong nucleophile. Initiation involves the addition of the initiator to the double bond of the monomer. The reaction produces a carbanion at the head end to which is associated the positively charged counterion. Propagation occurs by the successive insertion of monomer molecules by anionic attack of the carbanion. Termination of the growth activity of the polymer chain takes place either by deliberation or by the introduction into the system of oxygen, carbon dioxide, methanol, water, or other molecules that are capable of reacting with the active chain ends. In anionic polymerization as well as in free-radical polymerization, the initiator or part of it becomes part of the resulting polymer molecule attached to a nongrowing chain end.⁵

2.3.2.4 Polymers Synthesized by Ionic Polymerization

2.3.2.4.1 Polyisobutylene

Isobutylene polymerizes in the presence of a catalytic amount of BF_3 . The polymerization reaction takes place according to a cationic polymerization mechanism (Scheme 2.16).⁵

The catalyst, BF_3 , forms a complex with water to yield an active proton, $H^+(BF_3OH)^-$. Further, the complex combines with an isobutylene molecule to form a carbonium ion. The carbonium ion reacts with isobutylene molecules

Initiation

 $\mathsf{BF}_3 + \mathsf{H}_2\mathsf{O} \longrightarrow \mathsf{H}^+(\mathsf{BF}_3\mathsf{OH})^-$



Propagation





Termination



SCHEME 2.16

to form a long chain of target molecular weight polymer. From the long chain of carbonium a proton is given out for the formation of polyisobutylene to stop the reaction.

2.3.2.4.2 Polymethacrylonitrile

Methacrylonitrile polymerizes by a base catalyst. The polymerization reaction takes place according to the anionic polymerization mechanism (Scheme 2.17).⁵

The potassium amide ionizes to a potassium and amide ion $(-NH_2)$. The negatively charged amide ion further reacts with a methacrylonitrile molecule to form a carbanion. The carbanion reacts with methacrylonitrile molecules to form a long chain of target molecular weight polymer. When the polymer chain reaches the target molecular weight, the carbanion abstracts a proton from the ammonia molecule to stop the polymerization reaction and form polymethacrylonitrile.

2.4 RING-OPENING POLYMERIZATION

The process in which the polymerization takes place with the opening of a substrate ring is called ring-opening polymerization. The main advantages of

Initiation

 $KNH_2 \longrightarrow K^+ + NH_2^-$

$$H_{2C} \stackrel{\text{CH}}{=} H_{12} \stackrel{\text{H}}{=} H_{2N} \stackrel{\text{CH}}{=} H_{2N} \stackrel{\text{C$$

Propagation



Termination



Ĥ.

SCHEME 2.17

this polymerization process over the step-growth polymerization are the ability to form high molecular weight polymers without the formation of by-products. Glycolide, lactides, and ϵ -caprolactone are mostly used as monomers for the synthesis of biodegradable polymers by ring-opening polymerization using some efficient catalyst systems.

In ring-opening polymerization, the polymers have been produced from cyclic organic compounds including epoxides such as ethylene and propylene oxide, epichlorohydrin, and other cyclic ethers like trioxane and tetrahydrofuran. The other important cyclic compounds involved in ring opening polymerization are cyclic amides (lactams), cyclo-olefins, and siloxane.

Ring-opening polymerization involves an initial ring opening of a cyclic monomer followed by addition of rings to form a polymer chain. The ringopening polymers are normally linear having the same composition as the monomer but in an open chain. The major applications of these polymers are in coatings, fibers, elastomers, adhesives, and thermoplastic and thermoset-based composite systems.

The ring-opening polymerization proceeds by anionic or cationic catalyst, but usually not by radicals. This is shown in Scheme 2.18 where X is the heteroatom such as O or S, or a group like NH, -O-CO, -NH-CO or -C=C.

The nature of X in a cyclic compound should be such that it provides a mechanism for a catalyst or initiator to form the initiating coordination intermediate with the cyclic ring.⁵



SCHEME 2.18

2.4.1 MECHANISM AND KINETICS OF RING-OPENING POLYMERIZATION

The ring-opening polymerization reaction involves an alkylidene catalyst. The reaction takes place in two steps. In the first step the new olefin generated remains attached to the catalyst with a generic strained cyclic olefin. In the second step the generated strain is responsible for the opening of a ring, which is an irreversible step (Scheme 2.19).



SCHEME 2.19

2.4.1.1 Kinetics

In kinetic analysis,⁸ it is generally assumed that the rate of reaction can be described by two seperable functions, K(T) and, $f(\alpha)$, such that

$$\frac{\mathrm{d}\alpha}{\mathrm{d}t} = K(T)f(\alpha) \tag{2.46}$$

where $d\alpha/dt$ is the rate of reaction, K(T) is the temperature-dependent rate constant, and $f(\alpha)$ corresponds to the reaction. The temperature dependence of the reaction rate is commonly described by the Arrhenius equation:

$$K(T) = A \exp\left(\frac{-E}{RT}\right)$$
(2.47)

where R is the universal gas constant, E is the activation energy, and A is the pre-exponential factor.

For experiments, in which samples are heated at a constant rate, the explicit time dependence in Equation (2.46) can be eliminated so that

$$\frac{\mathrm{d}\alpha}{\mathrm{d}T} = \frac{A}{\beta} \exp\left(-\frac{E}{RT}\right) f(\alpha) \tag{2.48}$$

where $\beta = dT/dt$ is the heating rate.

A multivariate version of the Borchardt and Daniels method⁹ is frequently used in the evaluation of dynamic DSC data. In this method, the kinetic parameters (A and E) are obtained by a linearizing transformation of Equation (2.48) so that

$$\ln \frac{d\alpha/dT}{f(\alpha)} = \ln \left(\frac{A}{\beta}\right) - \frac{E}{RT}$$
(2.49)

This linear equation, which has the form $y = a_0 + a_1x$ with x = 1/T, can be used to determine the optimal fit of the kinetic parameters by multiple linear regression.

2.4.2 ANIONIC AND CATIONIC RING-OPENING POLYMERIZATION

Anionic polymerization of propylene oxide involves an attack of the least sterically hindered carbon by the hydroxyl anion to produce the alkoxide, which initiate the polymerization reaction for the formation of linear polymer molecules (Scheme 2.20a).

Cationic polymerization involves protonation of the oxide which activates the least sterically hindered carbon toward a nucleophilic attack of another propylene oxide (Scheme 2.20b).⁵

2.4.3 POLYMERS SYNTHESIZED BY RING-OPENING POLYMERIZATION

The hydrolytic polymerization of caprolactam produces Nylon 6. In this process, water opens the caprolactam ring, producing aminocaproic acid as a zwitterion. The zwitterion initiates step polymerization of the monomer forming a linear polymer molecule. In this way the polymerization involves an initial ring-opening of the monomer followed by a step polymerization (Scheme 2.21).⁵

This hydrolytic polymerization involves the heating of caprolactam in the presence of water at 250 to 270°C for 12 to 24 h. Most of the water used to initiate the reaction is removed during the process after 90 % conversion.

2.4.3.1 Enzyme-Catalyzed Ring-Opening Polymerization

Thermophilic biocatalyst enzymes have unique stability over a range of pH, temperature and solvent conditions, which permits their use in a wide range of



b. Cationic Polymerization



SCHEME 2.20



SCHEME 2.21

conditions without loss in enzymatic activity. They are also chemo and stereoselective. Ring-opening polymerization of racemic β -hydrolactone catalyzed by thermophilic lipases produces optically active *R*-enriched poly(3-hydroxy-butyrate).



SCHEME 2.22

In a typical polymerization, a mixture of β -hydrolactone and lipase ESL-001 was stirred in isooctane and heated in an oil bath at 60 to 80°C. Removal of solvent and enzyme from the reaction mixture resulted in a high molecular weight polymer (Scheme 2.22).

Due to the unique thermal stability of the enzyme, the polymerization could be carried out at 60 to 80°C, which reduces the reaction time. The water traces in the system act as an initiator. The mechanism of lipase catalysis involves the reaction of the enzyme with the lactone, forming an acyl–enzyme intermediate which then reacts with the hydroxyl group of a growing polymer chain to continue the propagation. The formation of *R*-enriched polymers can be attributed to the rate difference between of acyl–enzyme intermediate with *R*- or *S*-configuration of chain ends.⁶

2.5 DENDRITIC POLYMERS

Dendritic polymers are a novel class of macromolecules, distinguished from linear and randomly branched polymers by the inclusion of precisely one branch point per repeat unit. The polyamidoamine dendrimers are synthesized by the repetitive unit in two steps: Michael addition of methacrylate to the amine, followed by regeneration of amine termini with ethylene diamine.

The first synthesized dendrimers were polyamidoamines (PAMAMs). They are also known as starburst dendrimers (the term 'starburst' is a trademark of the Dow Chemicals Company, Midland, MI, USA). Ammonia is used as the core molecule where, in the presence of methanol, it reacts with methylacrylate. Ethylenediamine is then added (Scheme 2.23).

The unique behaviour of dendrimers is suitable for a wide range of biomedical and industrial applications. Many potential applications are based on their unparalleled molecular uniformity, multifunctional surface and presence of internal cavities.

- 1. Dade International Inc. (Deerfield, IL, USA) has introduced a new method in cardiac testing. Proteins present in a blood sample bind to immunoglobins which are fixed by dendrimers to a sheet of glass. Conjugates of dendrimer and antibody improve precision and sensitivity of the test.
- Dendrimers have been tested in preclinical studies as contrast agents for magnetic agents.
- 3. Dendrimers are used in the targeted delivery of drugs and other therapeutic agents. Drug molecules can be loaded both in the interior of the dendrimers as well as attached to the surface groups.

TABLE 2.2 Commercially important polymers prepared by ring-opening polymerization					
Polymer type	Polymer repeating group	Monomer structure	Monomer type		
Polyalkene		(CH ₂) _x CH	Cycloalkene		
Polyether			Trioxane		
Polyether	(CH ₂) _x O	(CH ₂) _x O	Cyclic ether		
Polyester	О С-О	(CH ₂) _x 0	Lactone		
Polyamide	$ \begin{array}{c} O \\ II \\ H \\ - \left(CH_2 \right)_x \\ C \\ - N \\ \end{array} $	(CH ₂) _x NH	Lactam		
Polysiloxane	$ \begin{array}{c} CH_3 \\ + S_{i-}O \\ CH_3 \end{array} \right) $	[Si(CH ₃) ₂] _x	Cyclic siloxane		
Polyphosphazene			Hexachloro cyclotriphosphazene		
Polyamine	-CH ₂ CH ₂ NH	$H_2C - CH_2$	Aziridine		

4. Dendrimers can act as gene carriers. Polyamidoamine dendrimers are a class of highly branched cationic polymers which are capable of condensing DNA and delivering it to a variety of cell lines with minimum cytotoxicity. They are terminated in amino groups which interact with phosphate groups of nucleic acids. This ensures consistent formation of transfection complexes. A transfection reagent called SuperFect consisting of activated dendrimers is commercially available.



SCHEME 2.23

Activated dendrimers can carry a larger amount of genetic material than viruses. SuperFect–DNA complexes are characterized by high stability and provide more efficient transport of DNA into the nucleus than liposomes. The high transfection efficiency of dendrimers may not only be due to their well-defined shape but may also be caused by the low pK of the amines. The low pK permit the dendrimer to buffer the pH change in the endosomal compartment. Starburst PAMAM dendrimers function as an effective delivery system for antisense oligonucleotides and antisense expression plasmids for the targeted modulation of gene expression.⁷

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3 Copolymers, Block Copolymers, and Stimuli-Sensitive Polymers

Kang Moo Huh, Sang Cheon Lee, Young Jin Kim, and Kinam Park

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3.1 INTRODUCTION

When only one type of monomer is used for polymerization, the resultant polymer consisting of the same repeating unit is called a "homopolymer." If polymerization is carried out with two different types of monomers (i.e., copolymerization), the polymer is known as a copolymer. When three different types of monomers are incorporated in the same chain, the term "terpolymer" is often used. It is noted that a copolymer is not a mixture of different homopolymers. In addition to traditional synthetic methods for copolymers (Figure 3.1), various novel synthetic methods have been developed to provide



FIGURE 3.1 Typical synthetic methods for copolymers.



FIGURE 3.2 Schematic representation of various types of homo- and copolymers.

better control on the structures and properties of the copolymers. By combining two or more monomers into one polymer chain, it is possible to obtain a new polymer with better properties than those of the individual homopolymers. Because of such flexibility in producing polymers with new properties, copolymers have been widely studied for their syntheses, characterizations, mechanical behavior, and applications.

Various kinds of copolymer structures are available as illustrated in Figure 3.2. Copolymers can be divided into four groups according to their structures. If two monomers or structural units are arranged in alternating fashion along a polymer chain, the polymer is known as an alternating copolymer. Most step-growth polymers are basically synthesized by condensation reaction of two types of monomers, and so they may be considered as

alternating copolymers. However, these are commonly treated as homopolymers with the repeating unit corresponding to the dimeric residue. If the monomers are randomly distributed along a copolymer chain, the polymer is called a random copolymer. This is the most frequently encountered structure. The term "random copolymer" has often been used interchangeably with "statistical copolymer."¹ A block copolymer is comprised of blocks (or segments) of individual monomers that are connected to each other. In a graft copolymer, polymer blocks are grafted as branches onto the main chain consisting of a different homopolymer.

3.2 PROPERTIES AND APPLICATIONS OF COPOLYMERS

3.2.1 GENERAL PROPERTIES OF COPOLYMERS

Over the past decades, there have been enormous successes in the synthesis and application of various copolymers. Copolymerization may offer the ability to alter the properties of a homopolymer in a desired way by the introduction of an appropriate second monomer. It is also possible to combine the desirable properties of two different homopolymers into a single polymer. For this reason, copolymerization has been used as a useful tool to make polymers with tailor-made properties which are critical for practical applications, such as crystallinity, flexibility, crystalline melting temperature (T_m) , and glass transition temperature (T_g) .

There are many factors influencing the properties of copolymers. In random copolymers, there is no orderliness to the arrangement of the two (or more) different monomers. Introducing the second type of monomers reduces the structural regularity of the polymer. Thus, the random copolymers have lower crystallinity than either of the respective homopolymers or become amorphous even if both homopolymers are crystalline. In general, the properties of random copolymers tend to have a weighted average of the homopolymers' properties. For example, the introduction of a comonomer into a polymer can change the T_g of the polymers. One simple relation, which is useful to describe the behavior of many vinyl monomer pairs, is:

$$1/T_g = w_A/T_{gA} + w_B/T_{gB}$$

where w_A and w_B refer to the mass fractions of monomers, A and B.² More complicated T_g equations for copolymers are introduced to theoretically calculate the T_g of copolymers available in other literature.³ However, there are often deviations from ideality observed in copolymer systems.

If a comonomer is introduced to a crystalline polymer, a marked decrease in crystallinity is usually observed. Such a decrease in crystallinity of a copolymer is obvious even with the introduction of a relatively small amount of the second comonomer, typically accompanied by reduction in stiffness, hardness, and softening point. In many cases, a rigid, fiber-forming polymer is converted to a highly elastic, rubbery product by such minor modification.



FIGURE 3.3 Changes of density and melting point as a function of the vinyl alcohol content in ethylene-vinyl alcohol (EVA) copolymers.

The mechanical strength of copolymers significantly depends on the copolymer compositions, which results primarily from changes in intermolecular forces as measured by cohesive energy. Higher cohesive energy results in higher stiffness and hardness and generally improved mechanical properties.⁴

Ethylene-vinyl alcohol (EVA) copolymers are random copolymers of ethylene and vinyl alcohol (VA), and they have been widely used for diverse applications. Their properties vary widely with the copolymerization composition.⁵ Figure 3.3 shows changes in density and melting point as a function of the VA content in copolymers. The polymer density increases nearly linearly with the VA content. The T_m value shows the minimum value of 107°C for EVA containing 13.8 mol% of VA. The presence of the minimum value is due to loose packing of the polymer chains by incorporation of hydroxyl groups. The increase in T_m for EVA copolymers with a VA content larger than 40.0 mol% is due to the formation of intermolecular hydrogen bonds. This tendency also corresponds to the change in the elastic modulus.

While the properties of random copolymers can be predictable to some degree, for other types of copolymers it is difficult to predict their properties. Many complicated factors exist that can affect the properties of copolymers. In general, random and alternating copolymers have characteristics which are the results of a compromise between the two different homopolymers, whereas block and graft copolymers retain the properties of the parent homopolymers. In the cases of block and graft copolymers are phase-separated, showing differentiated properties. One of the characteristic features of block copolymers is the existence of two T_g points that correspond to each polymer block.

3.2.2 PHARMACEUTICAL APPLICATIONS OF COPOLYMERS

Copolymers are very important polymeric materials because their properties can be tailor-made for specific applications. Copolymerization allows synthesis of almost unlimited number of different copolymer products, which are

prepared by variations in the types of monomers and the relative amounts of the monomers. For this reason, many commercial synthetic polymers are copolymers and have been extensively explored for pharmaceutical and biomedical applications. Copolymerization produces desirable properties for specific applications that cannot be demonstrated with corresponding homopolymers. For example, ethylene-vinyl acetate (EVAc) copolymers represent the largest-volume segment of the ethylene copolymer market. As the vinyl acetate (VAc) content increases, there are decreases in crystallinity, glass and crystalline melting temperatures, and chemical resistance coupled with increases in optical clarity.⁶ The EVAc copolymers are a heat processable, flexible and inexpensive material, and thus they have been investigated extensively as membrane or matrix systems for controlled drug release applications.^{7–10} The permeability property of the copolymers can be easily controlled by the copolymer composition or the vinyl acetate content, and thus it is possible to tailor a device with a desired release rate by only a small change in the membrane composition. The effect of the comonomer ratio on drug permeation was studied extensively using EVA membranes prepared from different vinyl acetate contents.⁷ The permeation profile of ethinylestradiol through the EVA copolymer membranes was examined as a function of the composition ratio of ethylene and vinyl acetate. An increase in the VAc content was observed to lead to increased drug release rate and permeability coefficient, because the introduction of VAc comonomers to a highly crystalline polyethylene decreases the crystallinity of the copolymer system, enhancing the diffusivity of polymer.

Biodegradable polymers have been extensively investigated for pharmaceutical applications, because they are degraded and removed from the body with no need for surgical removal after their use. Biodegradable polymers are also useful in the design of delivery systems for high molecular weight drugs, such as peptides and proteins, which are not suitable for diffusion-controlled release systems using non-degradable polymers. The copolymers of lactide (LA) and glycolide (GA), poly(lactic-*co*-glycolic acids) (PLGAs), are the most widely used family of biodegradable polymers. They have found a broad range of pharmaceutical and biomedical applications due to their unique advantageous properties, including versatile degradation kinetics, non-toxicity, and biocompatibility.^{11–13} As shown in Figure 3.4, PLGAs are usually synthesized by ring-opening copolymerization of cyclic monomers, L- or D,Llactide and glycolide, using various kinds of catalysts (mostly, stannous octoate), but also in the absence of catalysts.^{11,14,15}



FIGURE 3.4 Synthetic scheme of poly(L(or D,L)-lactide-co-glycolide).

Polyglycolide (PGA) is a highly crystalline polymer. It has a very high melting point (224 to 226°C) and its degradation rate is higher than that of PLA. Random PLGA copolymers have a broad spectrum of degradation rates, ranging from weeks to months, depending on the composition ratio of LA and GA. Thus, their biodegradability can be modulated to exhibit specific degradation kinetics according to their intended applications. They are generally more amorphous than their homopolymers and become most susceptible to hydrolysis when the two monomer contents are the same. There are a great number of copolymers developed for diverse applications and their number will continue to increase with increasing needs for new materials with improved properties and functionality.

3.3 BLOCK COPOLYMERS

3.3.1 GENERAL PROPERTIES OF BLOCK COPOLYMERS

Block copolymers are polymers composed of two or more polymeric blocks (or segments) connected to each other in the polymer chain. Recently, there has been increasing interest in block copolymers due to their unique properties as well as their broad technological applications. Block copolymers allow combining the properties of their constituent polymers in a unique way. Depending on the ratio, relative position, miscibility, morphology, and T_g of the different blocks, a wide variety of macroscopic properties can be achieved. The most commonly encountered block copolymer structures consist of a block of the first monomer covalently bonded to another block of the second monomer. These block copolymers are generally prepared by sequential anionic addition, ring-opening polymerization, or step polymerization of macromonomers with functional end groups.

Block copolymers are classified into several types based on sequential arrangement of component segments. Figure 3.5 shows various kinds of block copolymer structures. The simplest block copolymer is the AB type diblock copolymer, which is composed of one block of homopolymer A and the other block of homopolymer B (Figure 3.5a). In the second type of block copolymers, both ends of a B block are connected to each end of an A block, and thus, it is referred to as an ABA type triblock copolymer (Figure 3.5b). The BAB type triblock copolymers are obtained if the sequence of the blocks is reversed. In the third type of block copolymers, A and B blocks are arranged in an alternating fashion along the polymer chain. Such a block copolymer is called an alternating multiblock copolymer (Figure 3.5c). More than two blocks can be used to construct multiblock copolymers. The fourth type of block copolymers is multi-armed block copolymers (Figure 3.5d), which have two or more blocks connected at either or both ends of the other polymer block. When the blocks are introduced to the terminals of the first block having multifunctionality, polymers with a star-shape structure are formed (Figure 3.5e). In this case, the number of arms of the star-shaped block copolymers is determined by the number of functional end groups of the first polymer block.



FIGURE 3.5 Schematic presentation of various block copolymer structures.

The characteristics of block copolymers result largely from the fact that blocks of different compositions, constitutions, or configurations are mutually incompatible. Thus, different blocks tend to segregate into separate domains. At a temperature of usage, one domain is typically designed to be crystalline or glassy with high modulus to impart mechanical stiffness to the material, and such a domain is called hard segment. The other is then designed to be soft and so it retains good molecular flexibility in the material. This type of material has an elastomeric property between the softening temperatures of the soft and the hard segments. Above the softening temperature of the hard segments, however, the material starts to flow, and therefore it allows melt processing like a thermoplastic polymer. For this reason, these materials are called thermoplastic elastomers.

3.3.2 THERMOPLASTIC ELASTOMERIC BLOCK COPOLYMERS

Some of the most attractive block copolymers are the thermoplastic elastomeric block copolymers. They exhibit rubber-like elasticity without requiring any chemical crosslinking. Block copolymers consisting of both soft elastomeric segments with a low T_g and hard thermoplastic segments with a high T_g or high crystalline melting point, behave as thermoplastic elastomers (TPEs). The unique properties of thermoplastic elastomers can be attributed to the physical crosslinks (or physical junctions), which hold amorphous soft domains together as shown in Figure 3.6. The physical crosslinks can dissociate when heated above a certain temperature and reappear immediately upon



FIGURE 3.6 Typical structure of thermoplastic elastomers.

Soft segments	Hard segments	Block types	Reference
Poly(dimethylsiloxane)	Poly(ether keton sulfonate)	Multiblock	[16]
	Polyamide (aramide)	Multiblock	[17,18]
	Polystyrene	Triblock	[19]
		Multiblock	[19]
	Poly(L-lactide)	Multiblock	[20]
Polyisobutylene	Polystyrene	Diblock or star-shaped	[21]
		Triblock	[22]
Polybutadiene	Polystyrene	Triblock	[22]
Poly(tetramethylene oxide)	Poly(ethylene terephthalate)	Multiblock	[23]
Poly(<i>\varepsilon</i> -caprolactone)	poly(2,2-dimethyltrimethylene carbonate)	Triblock	[24]
Poly(ethylene glycol)	Poly(L-lactide)	Diblock	[25]
		Triblock	[26]
		Multiblock	[27]
	Poly(lactide-co-glycolide)	Multiblock	[28]

TABLE 3.1 Typical examples of soft and hard segments of block copolymers

cooling. Usually, the hard blocks are incompatible with the soft blocks, thus phase-separated on the microscopic scale.

Since it is necessary for the soft segments to be attached to more than two hard segments in order to ensure physical crosslinking, ABA triblock and (AB)*n* multiblock structures have been most frequently used to obtain elastomers. Some typical examples of segments making up of block copolymers that show thermoplastic elastomeric behavior are listed in Table 3.1.^{1,16–27}

For preparation of block copolymers in general, at least one of the segments has to be prepared separately by synthesis of end-functionalized oligomers. To obtain linear multiblock structures, the starting oligomers (telechelics) must have two functional end groups. Living ionic polymerization is one of the best ways to control molar mass, molar mass distribution, and number and nature of the end groups, and is therefore used widely. Step reaction polymerization also ensures the formation of two reactive groups at the ends of each polymer chain. However, in this case the nature of the end groups is predetermined only at complete conversion.

Other unique methods for synthesis of thermoplastic elastomeric block copolymers have been introduced. Maier et al.²⁸ have utilized the "criss-cross"cycloaddition of diisocyanates and azines. This reaction proceeds via a step growth reaction mechanism, but the nature of the intermediates ensures that polymer chains with two isocyanate end groups are exclusively formed, independent of conversion and initial stoichiometry. "Criss-cross"-cycloaddition of 4,4'-diisocyanatodiphenyl ether and 4-methoxybenzaldazine was used to synthesize α, ω -diisocyanate telechelics, and these precursors were reacted with different α, ω -dihydroxy functionalized aliphatic polyethers to produce segmented multiblock copolymers exhibiting elastomeric behavior. Chen et al.²⁶ have reported on a direct copolymerization of L-lactide (LLA) and ethylene oxide (EO) monomers using a wide range of organometallic catalysts, leading to multiblock structures. The multiblock segment length and molecular weight of the copolymers were regulated by varying reaction temperature, reaction time, reaction medium, and catalyst structure. It was described that the multiblock had segment lengths that were sufficiently long to produce distinct crystalline phases corresponding to PLLA and PEO.

3.3.3 PHARMACEUTICAL APPLICATIONS OF BLOCK COPOLYMERS

Most applications of block copolymers are based on their ability to selfassemble, in bulk or in selective solvents, into ordered nanostructures, with dimensions comparable to chain dimensions. Changing the molecular weight, chemical structure, molecular architecture, and composition of block copolymers allows manipulation of the size scale, type of ordering, and characteristics of the nanostructures. Over the past decades block copolymers have been used in various applications including adhesives and sealants, blending agents for use with different homopolymers for producing materials with desired characteristics, surface modifiers of fillers and fibers in order to improve dispensability in the matrix, surfactants for phase stabilization, viscosity improvers of lubricating oils, and membranes for desalination.²⁹

Recently, thermoplastic hydrogels have attracted a great deal of attention for unique applications in the biomedical fields. Thermoplastic hydrogels (or physical hydrogels) maintain their ordered structure by noncovalent cohesive interactions, such as hydrophobic interaction, stereocomplex formation, ionic complexation and crystallinity. The physical associations act as physical junction domains. Although noncovalent associations are reversible and weaker than chemical crosslinking, they allow solvent casting and thermal processing, and the resultant polymers often show elastic or viscoelastic properties.³⁰ Usually, this type of block copolymer consists of non-swellable hard segments and swellable soft segments of high flexibility. Hydrophobic biodegradable polymers, especially polylactide (PLA) and polycaprolactone (PCL), are usually favored as hard segments, and poly(ethylene oxide) (PEO) is most commonly used for soft segments due to high hydrophilicity and nontoxicity. The hard segments frequently crystallize to cause microphase separation into domains of hard and soft segments. Thermoplastic hydrogels have several advantages, such as processibility and efficient drug loading, over thermosetting hydrogels (chemical hydrogels). In addition, thermoplastic hydrogels show high mechanical properties even in a swollen state while thermosetting hydrogels do not. The multi-component nature of the polymeric systems provides both biodegradation and good mechanical properties.

A number of biodegradable (or nondegradable), water-insoluble polymers have been copolymerized with hydrophilic polymers to form thermoplastic hydrogels, which are able to absorb considerable amounts of water without dissolving. Bae et al. have developed various thermoplastic hydrogels based on multiblock structures consisting of a hydrophobic polymer block (e.g., PLA, polv(tetramethylene oxide), poly(dimethyl siloxane) and poly(ɛ-caprolactone)) and a hydrophilic poly(ethylene oxide) block for biomedical and other applications.^{30–33}

Water-soluble amphiphilic block copolymers having large solubility differences between hydrophilic and hydrophobic segments can self-assemble into spherical micelles in water (Figure 3.7). These block copolymer micelles are able to provide a much wider range of applications than the micelles made of low molecular weight surfactants, especially as nano-scale drug delivery systems. They have a unique core-shell structure in water, where hydrophobic segments are segregated from the aqueous exterior to form an inner core surrounded by a palisade of hydrophilic segments. The hydrophobic inner core can be utilized to entrap and deliver hydrophobic drugs. The structureproperty relationships and applications of block copolymer micelles have been studied and reviewed in many articles.^{34–36} Recently, novel polymeric micelles that were tailor-made to deliver specific poorly soluble drugs such as paclitaxel were developed in our laboratory. The structure of the inner core of polymeric



Polymeric micelles in aqueous media


micelles was designed to increase the water-solubility of paclitaxel based on the low molecular weight hydrotropic agents, such as picolylnicotinamide and N', N'-diethylnicotinamide.^{37,38} Such hydrotropic polymeric micelles increased the solubility of paclitaxel by several orders of magnitude, and the stability of the hydrotropic micelles was maintained for months.

In addition to application in drug delivery, other potential applications of block copolymers include removal/recovery of organic/inorganic compounds from contaminated waters and nano-scale patterning based on their selfassembling nature.

3.4 STIMULI-SENSITIVE POLYMERS

3.4.1 GENERAL PROPERTIES OF STIMULI-SENSITIVE POLYMERS

Stimuli-sensitive polymers represent a rapidly growing area with enormous technological and commercial potentials in pharmaceutical and biomedical fields. Impetus for innovation has come from the need for smart materials that can change their structure and functions responding to various environmental stimuli such as pH, temperature, electrical field, ionic strength, salt type, solvent, and light. These smart materials, known as "stimuli-sensitive (or responsive) polymers" or "intelligent polymers," are useful to create unique functional polymeric systems mimicking the natural feedback systems, which can sense one or more stimuli and change their structures and functions in direct response to the stimuli.³⁹ Their responses are often accompanied by dramatic changes in shape, surface characteristics, solubility, molecular self-assembly, and sol-to-gel transition. A few examples of such changes are shown in Figure 3.8.⁴⁰



FIGURE 3.8 Aqueous behaviors of stimuli-sensitive polymers and hydrogels.

The unique behaviors of stimuli-sensitive polymers are based on intra- or intermolecular interactions of polymer chains. Generally, fundamental driving forces for stimuli-sensitive properties result from competition among van der Waals interaction, hydrophobic interaction, hydrogen bonding, and electrostatic interaction. Hydrophobic interaction between polymer chains or hydrophobic moieties that are chemically introduced to the polymers has been most frequently used to create stimuli-sensitive systems, and often modulated through chemical modification or copolymerization. In many cases, two or more kinds of interactions work cooperatively.

Stimuli-sensitive hydrogels can be prepared by simply crosslinking stimulisensitive polymers. The hydrogels have the same sensitivity toward stimuli as the polymers do. In general, the stimuli can be divided into three classes: physical (e.g., temperature, ionic strength, solvent, UV/VIS light, electric field, and magnetic field); chemical (pH, specific ions, and chemical species); and biological (enzyme, glucose, antigen, and other biomolecules).

3.4.2 THERMOSENSITIVE POLYMERS

Temperature is the most widely utilized stimulus in modulating delivery of therapeutic agents. Naturally, thermosensitive polymers and hydrogels are the most commonly studied class of stimuli-sensitive polymers for modulated drug delivery and other applications. Certain water-soluble polymers exhibit a phase transition known as a lower critical solution temperature (LCST) when the temperature is raised. This type of polymer is water-soluble below the LCST but precipitates from solution as the temperature increases above the LCST. Poly(*N*-isopropyl acrylamide) and its copolymers are representatives of such thermosensitive polymer schibiting the LCST phenomena. The phase diagram of the polymer solution exhibiting the LCST is shown in Figure 3.9. This LCST phenomenon of water-soluble polymers arises from the delicate balance between hydrophilicity and hydrophobicity of the polymers. The hydrophilic and hydrophobic balance can be obtained from either the repeating units of a homopolymer, random copolymers of monomer units with different degrees of hydrophilicity, or block copolymers of hydrophilic and hydrophobic blocks.



FIGURE 3.9 Aqueous phase diagram of poly(N-isopropyl acrylamide) with LCST.

The balance can also be controlled by various chemical methods, such as copolymerization, which significantly affects the thermosensitive properties of the polymers.

Most thermo-sensitive polymers are based on poly(N-alkylacrylamide), poly(vinyl methyl ether), and poly(N-vinylisobutylamide). The common feature of these polymers is that their monomeric structure retains both hydrophilic and hydrophobic parts. Among them, poly(*N*-isopropylacrylamide) (PNIPAAm) and its copolymers are representatives of most widely investigated thermosensitive polymers for a broad range of applications. PNIPAAm homoand copolymers are water-soluble, but become water-insoluble (or precipitates) above certain temperatures. Such phase transition phenomena are reversible and are caused by the dehydration of the hydrophobic isopropyl groups. The LCST of PNIPAAm is known to be around 32°C.⁴¹ The LCST of the polymer can be controlled through copolymerization with other hydrophilic (e.g., acrylic acid, N,N-diethylaminoethyl methacrylate, acrylamide) and/or hydrophobic comonomers (e.g., butyl methacrylate). Introduction of less hydrophobic comonomers tends to increase the LCST of the resulting copolymers, while the more hydrophobic comonomer enhances hydrophobic interaction, leading to lowering the LCST of the copolymers.

Thermosensitive hydrogels, which are crosslinked forms of thermosensitive polymers, demonstrate a dramatic volume phase transition in response to small changes in temperature. This property has been widely exploited in development of controlled drug delivery systems, biocatalyst immobilization matrices, and bioseparation.^{42–45} Okano et al. used PNIPAAm copolymers to demonstrate a reversible switching function corresponding to hydration/ dehydration of polymer chains in response to temperature changes for various biomedical applications.^{39,46–53} PNIPAAm copolymers have been attached to solid surfaces, cross-linked hydrogels, and biomolecules to create new PNIPAAm-grafted surfaces, comb-type-grafted hydrogels, and modified thermosensitive bioconjugates, respectively.

Several amphiphilic block copolymers with well-defined block structures and lengths have been found to exhibit unique thermosensitivity, such as LCST phenomenon, sol–gel transition, and swelling change. Poly(ethylene oxide)– poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) triblock copolymers (with commercial names such as Pluronics or Poloxamers) are thermosensitive block polymers. A variety of block structures that have different hydrophilicity or hydrophobicity are commercially available and their thermosensitive property has been investigated. They can exhibit a broad range of LCSTs ranging from 10°C to above 100°C depending on the block composition and structure and also exhibit a sol–gel transition in concentrated solutions.⁵⁴

PEO-PPO-PEO block copolymers are not biodegradable. For this reason, biodegradable block copolymer systems showing thermosensitivity have recently attracted much attention. Low molecular weight di- or triblock copolymers of biodegradable polyesters such as PLA and PLGA and hydrophilic PEO exhibit thermosensitive sol-gel transition and have been

investigated as a candidate for in situ forming hydrogel delivery systems.^{55,56} In addition, graft and multiblock copolymers have been found to show thermosensitivity.^{31,57,58} These block copolymer systems can be loaded with drugs in aqueous phase at low temperature (below the critical gelation temperature) where it forms a solution. Just after subcutaneous injection, temperature increase to 37°C (above the critical gelation temperature) converts the injected solution to a gel that can act as a sustained release matrix for the loaded drugs. Thermosensitive polymers are highly useful due to the simplicity of drug formulation by solution mixing, biocompatibility with biological systems, and convenient administration.

Other thermosensitive block copolymers have been developed based on a balanced structure between hydrophilic and hydrophobic blocks. Allcock et al. have reported the synthesis of diblock copolymers of poly[bis(methoxyethoxyethoxy)-phosphazene] and PEO and their thermosensitive behaviors.⁵⁹ Song et al. have developed a new class of thermosensitive cyclotriphosphazenes that are synthesized by stepwise substitution of hexachlorocyclotriphosphazene, (NPCl₂)₃, with alkoxy poly(ethylene glycol) (APEG) and amino acid esters (AAE).⁶⁰ These trimeric derivatives exhibit well controlled LCST properties than the conventional thermosensitive polymers by changing the composition and type of hydrophilic and hydrophobic substituents.

3.4.3 OTHER STIMULI-SENSITIVE POLYMERS

Polymers containing charged groups in their structures are pH-sensitive. Typical charged groups include carboxylic, sulfonate or sulfate, and primary or tertiary amino groups. The ionization/deionization process of cationic or anionic groups in polymers usually cause pH-sensitive transitions of polymer conformation, solubility, and swelling in aqueous solutions. The typical examples of pH-sensitive polymers are poly(acrylic acid), poly(methacrylic acid), polyethyleneimine, poly(L-lysine), and poly(N,N-dimethylaminoethyl methacrylamide). The pH in the stomach (pH = 1–2) is quite different from the neutral pH in the intestine. Hence, the gastrointestinal tract (GI tract) is an important site for applications of pH-sensitive polymers.⁶¹

In addition to the charged groups listed above, the sulfonamide group was used to create new pH-sensitive polymers having a sharp phase transition at around pH 7.4.^{62,63} Such new polymers can also form pH-sensitive nanoparticles with a sharp association/dissociation around the physiological pH. The transition pH can be controlled by copolymerization. These polymers are useful in designing polymers and hydrogels that require a transition in solubility and swelling at a desired pH.

Electric-sensitive and light-sensitive polymers have been investigated extensively for applications in micro-systems, tissue engineering and medical imaging. Electric field is easily adjustable using a relatively small and simple electric circuit. A number of polymeric systems that can respond to electric field have been developed. For example, a partially hydrolyzed polyacryla-mide gel undergo a phase transition upon application of an electric field.⁶⁴

Light-sensitive polymers have potential applications in developing optical switches, display units, ophthalmic drug delivery devices. The UV-sensitive hydrogels were synthesized by introducing a leuco-derivative molecule, bis(4-di-methylamino) phenylmethyl leucocyanide, into the polymer network.⁶⁵

Recently, biological stimuli-sensitive systems that can respond to specific biomolecules, such as glucose, enzymes, or antigens, have become increasingly important due to their potential applications in the development of biomaterials and drug delivery systems. Glucose-sensitive hydrogels were used to develop modulated insulin delivery systems.^{66,67} Miyata et al. reported an antigen-sensitive hydrogel prepared by grafting the antigen and corresponding antibody to the polymer network. Binding between the two introduces crosslinks in the network. Competitive binding by free antigens triggers a change in gel volume due to breaking of these non-covalent crosslinks. The hydrogel displays shape-memory behavior and stepwise changes in antigen concentration can induce pulsatile permeation of a protein through the network.⁶⁸ Several biological stimuli-sensitive polymers and hydrogels, including glucose-sensitive, protein-sensitive, and other biomolecules-sensitive systems, have been reviewed in the literature.^{69–71}

Some stimuli-sensitive polymers have been developed to respond more than two stimuli. Most existing stimuli-sensitive polymers are designed to be sensitive only to a single stimulus, while more sophisticated delivery systems may require more delicate polymeric systems that can function in response to many kinds of stimuli simultaneously or sequentially.

More sophisticated methods have been developed to create novel stimulisensitive hydrogels by specific non-covalent interactions. Tirrel et al. demonstrated reversible hydrogels from self-assembling proteins, which undergo reversible gelation in response to temperature or pH changes, using recombinant DNA methods.⁷² Yui et al. proposed supramolecular-structured hydrogels showing a thermosensitive gel–sol transition by reversible inclusion complexation between polymeric guests and α - or β -cyclodextrins as host molecules.^{73,74} They also demonstrated a pH- and/or temperature sensitive supramolecular assembling system using a specific host–guest interaction of cyclodextrin-conjugated polyelectrolytes.^{75,76}

3.4.4 APPLICATIONS OF STIMULI-SENSITIVE POLYMERS

Over the past two decades, stimuli-responsive polymers and hydrogels have been applied in the pharmaceutical as well as non-pharmaceutical fields. In addition to applications in controlled or modulated drug delivery, stimuli-sensitive polymers have been used in bioseparation, in the development of new biocatalysts, biomimetic actuators, and switchable hydrophilic–hydrophobic surfaces.^{77,78}

Various pharmaceutical applications of thermosenstive polymers and hydrogels are shown in Figure 3.10. Many review papers on their applications are available in the literature.^{39,40,47} Recent application of thermosensitive polymer in gene delivery utilizes PNIPAAm copolymers that allow controlled



FIGURE 3.10 Thermosensitive polymers for pharmaceutical applications.

formation/dissociation behavior of polymer–DNA complexes in each intracellular step such as uptake and transcription.⁴⁹

pH-sensitive polymers have also been used in making biosensors and permeation switches.⁷⁹ For these applications, they are usually loaded with enzymes that change the pH of the local microenvironment inside the polymers. Electric-sensitive polymers are basically pH-sensitive polymers. They are able to convert chemical energy to mechanical energy.⁸⁰ These systems can serve as actuators or artificial muscles in many applications. Light-sensitive polymers can be used in the development of photo-responsive artificial muscles, switches, and memory devices.⁸¹ Crosslinked hyaluronic acid hydrogels that undergo photosensitized degradation in the presence of methylene blue allows potential applications in visible light-responsive temporal drug delivery.

While the concepts of stimuli-sensitive polymers are promising for many applications, the practical uses require significant improvements in the polymer properties. The most significant weakness of all these stimuli-sensitive systems is that their response time is too slow. Thus, many current studies focus on fast-acting polymeric systems that are necessary for practical uses. A recent book focuses on the fast-responsive natural as well as synthetic systems.⁸² One approach of making fast responsive systems is making hydrogels thinner and smaller. Since there is a limit in making hydrogels thinner and smaller, alternative approach of making superporous hydrogels (SPHs) was developed.⁸³ The superporous hydrogels with good mechanical strength can be prepared by making SPH composites and interpenetrating SPH networks.^{84–86} Due to continuous advances in polymer chemistry, various fast-reacting stimuli-sensitive polymers and hydrogels designed for specific applications are expected to be developed in the very near future.

3.5 CONCLUDING REMARKS

Copolymers and stimuli-sensitive polymers are widely used in numerous applications. Stimuli-sensitive polymers experience changes in their structure and/or chemical properties in response to changes in environmental stimuli, such as temperature, pH, light (ultraviolet or visible), electric field, or certain chemicals. Volume changes of stimuli-sensitive gel networks are particularly responsive to external stimuli, but swelling is slow to occur, which will facilitate slow release of a given drug. pH-sensitive polymers are suitable candidates for oral delivery of peptide and protein drugs, since they will prevent gastric degradation in the stomach while providing a controlled release of a peptide or protein drug.

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4 Polymer Solution Properties, Micelles, Dendrimers, and Hydrogels

Tooru Ooya and Kinam Park

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References

4.1 INTRODUCTION

Water-soluble polymers have been extensively studied for biomedical and pharmaceutical applications.¹ The water-soluble polymers come originally from natural sources, including polysaccharides and proteins. Others are synthetic polymers such as poly(ethylene glycol) (PEG), poly(vinyl pyrrolidone) (PVP),



FIGURE 4.1 Illustration of various macromolecular architecture: (a) block copolymer, (b) comb-type polymer, (c) star polymer, (d) hyperbranched polymer and (e) dendrimer.

poly(vinyl alcohol) (PVA), poly(ethylene imine) (PEI), poly(acrylic acid) (PAA) and poly(2-methacryloyloxyethylphosphorylcholine). These watersoluble polymers show linear architectures that can hydrate and randomly distribute in aqueous solution. Recent advances of radical polymerization such as atom transfer radical polymerization (ATRP),^{2–5} reversible addition-fragmentation transfer (RAFT) polymerization,^{6,7} and nitroxide-mediated polymerization (NMP)^{8,9} have provided tailor-made synthesis of various macromolecular architecture, the representatives of which include block-, comb-type, star, and hyprerbrached polymers (Figure 4.1a–d).

In addition to the controlled radical polymerization methods, multifunctional molecules can be modified step-by-step to produce *dendrimers* (Figure 4.1e).¹⁰ Molecular weights of these macromolecular architectures can be increased with degree of polymerization, but their solution properties are quite different from those of linear architecture. Even at the same molecular weight, different local density of molecular chains leads to unique hydration and hydrodynamic properties. Further increase in the molecular weight by physically and/or chemically, leads to network formation to show hydrogel properties. Understanding the solution properties of new macromolecular architecture will help to develop the new polymeric architecture and hydrogels for protein and nucleic acid delivery, which have never been achieved by conventional polymers and hydrogels. In this chapter, polymer solution properties of branched polymers, micelles and dendrimers are introduced in terms of characterization methods and recent pharmaceutical applications.

4.2 CHARACTERIZATION METHODS OF SOLUTION PROPERTIES

4.2.1 VISCOSITY MEASUREMENTS

Viscometry has been utilized for characterization of solution properties of polymer solutions in terms of the size and shape in diluted conditions.



FIGURE 4.2 Typical example of Huggins plot.

Especially, associate properties between block and graft copolymers could be estimated by dilute solution viscosity. Capillary viscometry is an apparently simple technique that has been widely used to analyze polymers in diluted conditions. Viscosity is easily determined by the time t_0 (for solvent) and t (for polymer solution with different concentration) using various types of viscometer at constant temperature. From the results, the viscometric terms can be calculated by the following equations:

$$\eta_r = \eta/\eta_0 \approx t/t_0 \tag{4.1}$$

$$\eta_{sp} = \eta_r - 1 = (\eta - \eta_0) \approx (t - t_0)/t_0 \tag{4.2}$$

$$\eta_{red} = \eta_{sp}/c \tag{4.3}$$

where η_r is relative viscosity, η_{sp} is specific viscosity, η_{red} is reduced viscosity and *c* is the concentration of the polymer given in g/mL. In a dilute solution, the obtained values of $\eta_{red/c}$ is linear in *c* and the intrinsic viscosity ([η]) may be obtained by extrapolating a plot of $\eta_{red/c}$ versus *c* to c = 0 (Figure 4.2). This relation has been described by a purely empirical equation by Huggins:¹¹

$$\eta_{red}/c = [\eta] + k'[\eta]^2 c \tag{4.4}$$

where k', the Huggins constant, has values around 0.35 in strongly solvating solvents. The $[\eta]$ is related to the size of macromolecules, and the Huggins constant is an important parameter of the interactions between the chains. The relation between $[\eta]$ and viscosity average molecular weight (M_n) can be represented by the Mark–Houwink relationship:¹¹

$$[\eta] = KM_n^a \tag{4.5}$$

where K and a are constants for a given polymer-solvent-temperature system. Comprehensive lists of K and a values can be referred if one uses fine polymers.¹² Theory predicts that it should lie in the range 0.8 < a < 1.0 for inherently stiff molecules such as cellulose derivatives and DNA.

4.2.2 LIGHT SCATTERING MEASUREMENTS

Static and dynamic light scattering (SLS and DLS, respectively) measurements are a powerful tool for characterizing solution properties of polymers. Light scattering in dilute solutions are detected by SLS measurements and passing through filters with appropriate pore size is necessary to remove any dust. The weight average molecular weight (M_w) , second virial coefficient (A_2) , and the radius of gyration (R_g) of polymers were obtained from plots of $(KC/R(\theta))$ vs. $\sin^2(\theta/2)$ (Zimm plot):

$$\left[\frac{KC}{R(\theta)}\right]_{\theta \to 0} = \frac{1}{M_w} + 2A_2C = \frac{1}{M_w} \left(1 + \frac{16\pi^2 n_0^2}{3\lambda_0^2} \langle S^2 \rangle \sin^2\left(\frac{\theta}{2}\right)\right) + A_2C \qquad (4.6)$$

where $K = 2\pi^2 n_0^2 (dn/dc)^2 / N\lambda^4$ is the usual optical constant factor, n_0 is the refractive index of the solvent, N is Avogadro's number, λ_0 is the wavelength of light, $\langle s^2 \rangle$ is the z-averaged radius of gyration, C is the concentration, and $R(\theta)$ is the Rayleigh ratio. In the case of lower molecular weight, the M_w and A_2 are obtained by $(Kc/R(\theta))$ vs. C (Debye plot):

$$\frac{KC}{R(\theta)} = \frac{1}{M_w} + 2A_2C \tag{4.7}$$

Polymers avoid one another as a result of their van der Waals "excluded volume" when the A_2 value is large, and they attach to one another in preference to the solvent when it is small or negative.^{13,14} The general formula¹⁵ for the photoelectron count time correlation function is as follows:

$$g^{(2)}(\tau) = 1 + \beta |g^{(1)}(\tau)|^2 = 1 + \beta \exp(-2\Gamma\tau)$$
(4.8)

where $g^{(2)}(\tau)$ is the normalized second-order correlation function, β is a nonideality factor which accounts for the deviation form ideal correlation, $g^{(1)}(\tau)$ is the normalized first-order correlation function, τ is the delay time, and Γ is the relaxation rate. $g^{(1)}(\tau)$ can be expressed as Laplace transform of the distribution of relaxation rates, $G(\Gamma)$:

$$g^{(1)}(\tau) = \int G(\Gamma)(-\exp\Gamma\tau) \,\mathrm{d}\Gamma \tag{4.9}$$

In the DLS analysis, the autocorrelation functions are analyzed using the method of cumulants together with the following equation:

$$g^{(1)}(\tau) = \exp\left[-\Gamma\tau + (\mu_2/2)\tau^2 - (\mu_3/3!)\tau^3 + \cdots\right]$$
(4.10)

where μ_2/Γ^2 is a variance (polydispersity index).

In the cumulant approach, the z-averaged transitional diffusion coefficient, D_T , is obtained from Γ , according to the following equation:

$$\Gamma = D_T K^2 = 4\pi n \sin(\theta/2)/\lambda \tag{4.11}$$

In the dilute concentration region, the concentration dependence of D_T can be expressed by a first-order expansion:

$$D_T + D_0(1 + k_d C) \tag{4.12}$$

where D_0 is the transitional diffusion coefficient at infinite diffusion, and k_d is the diffusion second virial coefficient. The corresponding hydrodynamic radius, R_h , can be calculated by the Stokes–Einstein equation:

$$R_h = k_B T / (6\pi \eta D_0) \tag{4.13}$$

where k_B is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity.

The size distribution was estimated from the correlation function profile by the histogram analysis software. In the histogram method, Equation (4.2) is replaced by

$$g^{(1)}(\tau) = \int G(\Gamma_i) \exp(-\Gamma\tau) \Delta\Gamma$$
(4.14)

and $G(\Gamma_i)$, which is the distribution according to the ratio of light scattering by the solution with Γ , was determined by using Marquart nonlinear least squares routine. $G(\Gamma_i)$ is then converted into the particle size distribution G(d), using Equations (4.11) and (4.13). The distribution according to the weight ratios and the number ratios is then determined from G(d).

4.3 BRANCHED POLYMERS

4.3.1 CHEMISTRY AND CHARACTERIZATION

Branched polymers, including comb-type polymers (Figure 4.1b), star-shaped polymers (Figure 4.1c), and hyperbranched polymers (Figure 4.1d), are some of the topological polymers that had been investigated in the field of polyolefins from the 1920s to 1970s. Comb-type polymers are comprised of blocks of one chain grafted onto the backbone of the other as branches.¹⁶ These polymers usually have a molecular weight distribution due to the molecular weight distribution of the backbones or of the branches. Hydroxyl groups of polysaccharides have been used as initiating groups to polymerize some monomers as branched chains.¹⁷ Coupling reactions between hydroxyl groups of polysaccharides and L- or D-lactides using an activating agent



FIGURE 4.3 Coupling reactions between hydroxyl groups of polysaccharides and oligomeric graft chains using an activating agent (N,N'-carbonyldiimidazole).

(N,N'-carbonyldiimidazole) is another approach to synthesize comb-type copolymers (Figure 4.3).¹⁸ Hydroxyl groups of the polysaccharides can be activated by *p*-nitrophenyl chloroformate and then coupled with functional polymeric chains such as semi-telechelic poly(*N*-isopropylacrylamide)¹⁹ and amino-terminated PEG.²⁰

The branched structure of comb-type copolymers is usually confirmed by SLS and viscosity measurements. Conventional size exclusion chromatography (SEC) analysis is not suitable for determination of M_w of the comb-type polymers. The branched structure reduces the size of the polymer in comparison with the size of linear polymers. Thus, it always underestimates the M_w due to their smaller hydrodynamic volume in solution compared with linear polymer references such as PEG, Combination of SEC and SLS (SEC–SLS) is a powerful method to characterize both the real M_w and hydrodynamic volume. SLS measurements give the M_w and the root-mean-square radii of gyration (R_g) using a Zimm or Debye plot. R_g represents physical properties depending on molecular architecture and molecular weight. The reduction of R_g due to branching is by the following equation:

$$g = \langle R^2 \rangle_{br} / \langle R^2 \rangle_{lin}, \quad 0 < g < 1 \tag{4.15}$$

where the subscripts *br* and *lin* are the branched and linear polymer with the same molecular weight. Parallel to equation (4.15) a viscosimetric branching factor $(g_{[\eta]})$ can be calculated by the ratio of intrinsic viscosities:

$$g_{[\eta]} = [\eta]_{br} / [\eta]_{lin} \tag{4.16}$$

Assuming that both backbone and branched chains have no polydispersity about comb-type polymer, g is given by the following equation:²¹

$$g = \alpha - 2\alpha(1 - \alpha)/p + (3p - 2)(1 - \alpha)^3/p^2$$
(4.16)

where p and α are the number of graft chains and the weight fraction of backbone in the comb-type polymer, respectively.

The molecular weight dependence of R_g is given by:²²

$$R_g = AM_w^{\alpha} \tag{4.18}$$

where A is a constant, and α correlates to the polymeric structure. Generally, stiff polymers are likely to be of a rod-like structure, so that R_g and α values become larger. A random coil structure leads to smaller R_g and α values although these values are larger than those of spherical molecules in solution. In this sense, R_g and α of comb-type polymers are lower than linear polymers. Both the R_g and α values are decreased with an increase in the number of the graft chain, indicating the structure of comb-type polymers. Values of intrinsic viscosity also show the evidence of comb-type polymers.

Star-shaped polymers (Figure 4.1c) can be synthesized by ATRP^{23,24} and RAFT.²⁵ For the synthesis of star-shaped polymers, one terminal of several polymers is covalently or noncovalently bound at one point like stars. For a monodispersed star having *f* Gaussian chain arms, which means having arms of the same chain length, *g* is given by

$$g = (3f - 2)/f^2 \tag{4.19}$$

Equation (4.19) applies only to the limit of long flexible chains. Equations (15) and (16) are also applied for star-shaped polymers. Star-shaped PEG-*co*-poly(lactic acid) (PLA) and PEG-*co*-poly(glycolic acids) (PLG) exhibit a smaller hydrodynamic radius compared to linear ones. Such an architectural modification significantly affects its hyd-olytic properties due to the differences in chain length, crystallinity, and morphology.²⁶

4.3.2 PHARMACEUTICAL APPLICATIONS OF BRANCHED POLYMERS

Kissel et al. have studied protein drug release from hydrolyzable microspheres consisting of polysaccharides grafted with PLA-*co*-polyglycolide (PLGA).²⁷ When bovine serum albumin (BSA) is loaded in the microspheres, BSA can be

continuously released by diffusion without any drug burst. Formation of a porous, water-filled structure with the surface erosion leads to zero-order release profiles. Similar drug release is also observed when star-shaped PEGco-PLGA is used. Retaining PEG chains during the initial 2 to 3 weeks leads to swelling of the microspheres in that period. Cumulative release of FITCdextran from the star-shaped block copolymers was higher than that of linear copolymers.²⁷ Continuous release of protein drugs may be advantageous for advanced therapeutics using bioengineered proteins.²⁸ Park et al. have developed comb-type and star-shaped graft polymers consisting of poly(ethylene glycol) with molecular weight 400 (PEG400) graft chains as a new solubilizer of poorly water-soluble drugs (Figure 4.4).²⁹ The star-shaped graft polymers have a glucose molecule in the center position. Paclitaxel (PTX) solubility in water was increased by three orders of magnitude. The solubilized PTX was released from the graft polymers and star-shaped graft polymers into the surrounding aqueous solution. The release rate could be controlled by the comb-type and star shape (Figure 4.5).

4.4 POLYMERIC MICELLES

4.4.1 CHEMISTRY AND CHARACTERIZATION

Polymeric micelles consist of amphiphilic block copolymers including multiblock copolymers, AB type di-block copolymers, and ABA type tri-block copolymers with hydrophobic core (A or B segment) and hydrophilic shell (A or B segment). For pharmaceutical applications, a biodegradable segment consisting of PLA and PLGA is often selected. Biodegradable polymeric micelles of di-block copolymers such as PEG-*b*-PLA³⁰ and poly(β -maleic acid)*b*-poly(β -alkylmaleic acid alkyl ester),³¹ and tri-block copolymers such as PEG*b*-PLGA-*b*-PEG^{32,33} are prepared by mixing polymers in aqueous solution or dialysis of organic solution such as dimethylformamide (DMF) and dimethylsulfoxide (DMSO) against water. Films and microspheres are prepared by general film casting methods and the water-in-oil-in water (W/O/W) double-emulsion technique, respectively.

Micellization is usually characterized by dye solubilization methods using 1,6-diphenyl-1,3,5-hexatriene (DPH) and pyrene, including determination of critical micelle concentration (CMC), SLS and DLS measurements, gel permeation chromatography, and NMR measurements. For the dye solubilization methods, the dye molecule is preferentially partitioned into the hydrophobic core of micelles with the formation of micelles, which causes an increase in the absorbance of the dye. By changing the concentration of the block copolymers, CMC can be determined as crosspoint of extrapolating the change in absorbance in a wide range of the concentration.³² The M_w of micelles is calculated by the results of static light scattering (SLS) measurements using the Debye plot:

$$K(C-CMC)/(R-R_{CMC}) = 1/M_w + 2A_2(C-CMC)$$
 (4.20)





FIGURE 4.4 Comb-type (a) and star-shaped graft polymers (b) consisting of PEG400 graft chains as a new solubilizer of poor water-soluble drugs.



FIGURE 4.5 Cumulative release profiles of paclitaxel that was solubilized in aqueous solutions containing 10 wt% of star-shaped PEG400 graft polymers (closed circle), 10 wt% of comb-type PEG400 graft polymers (closed triangle) and paclitaxel powder (closed and gray-colored square). Reproduced from Ref. 29 (Ooya et al. 2003), with permission.

where *K* indicates $4\pi^2 (dn/dc)^2/N_A \lambda^4$, *R* and R_{CMC} are the excess Raileigh ratios at concentration *C* and CMC, *n* is the refractive index of solution at CMC, dn/dc is the refractive index increment, N_A is the Avogadro number, λ is the wavelength of laser light, and A_2 is the second virial coefficient. The R_h of micelles can be determined by DLS measurements using Equations (4.11) and (4.13). Since polymeric micelles are a kind of spherical assembly, the diffusion coefficients are generally independent of detection angle due to the undetectable rotational motions.^{15,34}

4.4.2 PHARMACEUTICAL APPLICATIONS OF MICELLES

Stability of core-shell structure of polymeric micelles is likely to be greater than that of conventional surfactant-based micelles. Kataoka and Okano et al. have developed polymeric micelles composed of block copolymers of PEG and poly(amino acid)s for anticancer drug delivery.^{35,36} In this system, hydrophobic drugs, such as doxorubicin, distribute into the core part of block copolymer micelles, and the long circulation times of block copolymer micelles has been demonstrated. Block copolymers that form micelles accumulate passively at solid tumors and thus have great potential for anti-cancer drug delivery. Kataoka and his associates developed polymeric micelles (Figure 4.6)³⁷ for delivery of proteins^{38,39} and nucleic acids.^{40,41} These authors achieved glutathione (GSH)-sensitive stabilization of polyion complex (PIC) micelles entrapping antisense oligonucleotide (ODN) by reversible cross-linking of the core through disulfide bonds.⁴² The ODN in the core cross-linked micelles was



FIGURE 4.6 Features of polymeric micelles that are designed by Kataoka et al. Reproduced from Ref. 37, with permission.

stabilized against nuclease. One unique point of this ODN carrier is that the micelles dissociate to release ODN in the presence of GSH at a concentration comparable to the intracellular environment.

Jeong et al. studied the release of hydrophilic and hydrophobic drugs incorporated into PEG-*b*-PLGA-*b*-PEG-based polymeric micelles that act as hydrogels at body temperature.⁴³ A model hydrophilic drug is released with a first-order profile. On the other hand, a model hydrophobic drug is released with a sigmoidal release profile, suggesting that the drug release is initially governed by diffusion, and later followed by degradation of the micelles. This biphasic drug release is consistent with a simulation model of hydrophobic drug release from a core-shell structure of matrix in which the hydrophobic drug is partitioned into different domains. Since the polymeric micelles become a hydrogel at body temperature, such biodegradable and thermosensitive hydrogel formulations may be fascinating techniques for injectable drug delivery systems.⁴⁴

4.5 DENDRIMERS FOR PHARMACEUTICAL USE

4.5.1 CHEMISTRY AND CHARACTERIZATION

Dendrimers are nano-sized, highly branched macromolecules with monodispersed characters, and are derived from the Greek words *dendron* (tree) and *meros* (part). Due to the nano-sized spherical shape and multivalent surface functionalities, they are focused as a host of biomimetic and nanotechnological applications.¹⁰ Tomalia et al. have reported the successful synthesis of "starburst-dendritic macromolecules" in 1985.⁴⁵ The poly(amidoamine) (PAMAM) dendrimers are prepared by the divergent methodology based on acrylate monomers. Nowadays, the PAMAM dendrimes, named as Starburst[®] dendrimers, are commercially available from Aldrich (Milwaukee, WI). Another approach to preparing dendritic architecture is developed by Fréchet et al. who demonstrated the convergent growth approach with polyether dendrimers that are recognized as "Fréchet-type dendrimers."^{46,47}

Physical properties of dendritic architecture are quite different from the linear one in terms of conformations, crystallinity, solubilities, intrinsic viscosities, entanglements, diffusion, and electronic conductivity. With an increase in the generation, the dendrimers undergo congestion-induced molecular shape change from flat, floppy conformations to robust spheroids.^{48,49} The number of surface functional groups, Z, amplifies with the generation (G). The Z values are defied as:

$$Z = N_c N_b^G \tag{4.21}$$

where N_c is core multiplicity and N_b is branch cell multiplicity. According to Equation (4.21), the surface cells are amplified exponentially with the G, whereas the radii of the dendrimers increase in a linear manner. This relation leads to *dense-packed state*. Surface area per terminal groups can be calculated by the following equation:

$$A_z = \frac{A_D}{N_Z} \propto \frac{r^2}{N_c N_b^G} \tag{4.22}$$

where A_Z is the surface area per Z, A_D is the dendrimer surface area, and N_Z is the number of Z per generation. From Equation (4.22), it is easy to understand that at higher generations, A_Z becomes smaller and approaches van der Waals dimension of Z.

The intrinsic viscosity $[\eta]$ gives important information of solution properties of dendrimers. The $[\eta]$ is not continuously increased with molecular weight, whereas that of linear polymers are continuously increased (Figure 4.7).^{50,51} This indicates that the $[\eta]$ is not composition dependent. As mentioned above, the dendrimers become spherical shaped as a function of *G*, so that its volume grows by first approximation as n^3 (n = generation number), whereas the mass increases as 2^n . Since the $[\eta]$ is expressed in the ratio of a volume to a mass (d*L*/*g*), the value of $[\eta]$ should be decreased once a certain generation is reached.

Mass spectroscopy (MS) is an essential tool for characterization of dendrimers. Especially, MALDI-TOF MS analysis enables the theoretical mass to be compared with experimental values. Since the dendrimers have nearly complete monodispersity, the peak on MALDI-TOF spectra should be observed as only one peak. The other technology for analyzing unusual properties of dendritic states includes critical size exclusion chromatography,^{46,52,53} light scattering,⁵¹ atomic force microscopy,^{54,55} and gel/capillary electrophoresis.⁵⁶



FIGURE 4.7 Relationship between intrinsic viscosity and molecular weight for: (a) linear polymer, (b) hyperbranched polymer and (c) dendrimer. Reproduced from Ref. 50, with permission.

4.5.2 PHARMACEUTICAL APPLICATIONS OF DENDRIMERS

The PAMAM dendrimers have been extensively studied for potential use in a variety of therapeutic applications for human diseases.⁵⁷ The cationic surface of the PAMAM dendrimers has been subjected for complexation with anionic surface of DNA. The PAMAM dendrimers form complexes with DNA through sequence-independent electrostatic interactions. Charge neutralization of both components and alterations of the net charge of the complex lead to changes in both physicochemical and biological properties. Szoka et al. reported that PAMAM cascade polymers mediate transfection in a wide variety of cultured cells.⁵⁸ They found that the perfect dendritic structure (Starburst[®] dendrimer) shows a less effective transfection than the partially degraded dendrimers (fractured dendrimer).⁵⁹ Transfection activity is related both to the initial size of dendrimer and its degree of degradation that can be imaged by computer simulation (Figure 4.8). They suggested that high flexibility of the fractured dendrimer plays an important role in the mechanism of transfection. Toxicity of PAMAM dendrimers is dependent on both the concentration and generation. Malik et al. systematically studied biocompatibility and biodistribution of PAMAM dendrimes, and the results suggest that the amine groups located on the surface cause toxic problems.⁶⁰

An approach to enhance the biocompatibility of dendrimers involves conjugating PEG chain onto PAMAM dendrimer surface. Several researchers have tried to modify PAMAM dendrimers with PEG for biomedical applications. PAMAM dendrimers have been studied as a drug carrier. Wiwattanapatapee et al. showed that PAMAM dendrimers rapidly transferred serosal membrane aiming at oral drug delivery.⁶¹ The PAMAM dendrimers had potential as solubility enhancers of acidic drugs such as benzoic acid⁶² and ibuprofen⁶³ based on electrostatic interactions between the carboxyl groups of the drugs and amine groups of the PAMAM dendrimers. Kojima et al. developed PAMAM dendrimers having mono-methoxy PEG (M-PEG) grafts



FIGURE 4.8 Simulation model of fractured dendrimers: (a, left) 100% amide bonds remaining; (b, center) 50% amide bonds remaining; (c, right) 25% of amide bonds remaining. Reproduced from Ref. 59, with permission.



M-PEG-attached PAMAM dendrimer

FIGURE 4.9 Synthetic scheme of M-PEG attached PAMAM dendrimers. Reproduced from Ref. 64, with permission.

(Figure 4.9) to encapsulate adriamycin and methotrexate.⁶⁴ Adriamycin is a poorly water-soluble drug. These authors synthesized PEG-modified PAMAM dendrimers with different PEG chain length. It was found that the longer PEG chain was effective to encapsulate adriamycin in the PAPAM core. Luo et al. studied PEG-modified PAMAM dendrimers as a DNA carrier.⁶⁵ Here, the conjugated PEG chain reduced cytotoxicity of PAMAM dendrimers. Kobayashi et al. also synthesized PEG-modified PAMAM dendrimers as



FIGURE 4.10 Chemical structure of poly(glycerol-succinic acid) dendrimers. Reproduced from Ref. 68, with permission.

macromolecular MR contrast agents.⁶⁶ The PEG-modified PAMAM dendrimers remained in the blood significantly longer and decreased accumulation in the organ in comparison with PAMAM dendrimers. The PEG-modified PAMAM dendrimers also have also been studied as inorganic nanoparticles.⁶⁷ The PAMAM dendrimers have been extensively studied as hosts for encapsulated nano-sized metal or semiconductor particles, but the dendrimer–metal hybrid often aggregates in solution, resulting in the formation of multi-dendrimer cluster. By conjugating the PEG chain, the hybrid dendrimer could be soluble in various solvents. Moreover, PEGylation of dendrimers may also help in controlling the nanoparticle size.

Alternatively, biocompatible and/or biodegradable dendrimers based on poly(glycerol-succinic acid) (Figure 4.10)^{68–70} and polyester (Figure 4.11)^{71,72} have been used as drug delivery systems and tissue scaffolds. Polyglycerol dendrimers (PGDs)^{73,74} have good potential as biomaterials because of high water solubility, chemical reactivity, and structural similarity to PEG. Ooya et al. have developed the PGDs as "hydrotropic dendrimer" with generations 3 (G-3), 4 (G-4) and 5 (G-5) by step-by-step alkylation and dihydroxylation reactions (Figure 4.12).²⁹ The stability of paclitaxel in all the



FIGURE 4.11 Chemical structure of Dendritic polyester. Reproduced from Ref. 72, with permission.

solutions of PGDs even at low concentrations was much higher than those of PEG400, commonly used as a co-solvent or a hydrotropic agent. Paclitaxel solubility increased by PGDs in a dendrimer generation dependent manner. These results suggest that the dendritic structure contributed to the enhanced solubility even at low concentrations. ¹H NMR spectra of paclitaxel before and after mixing with PGDs in D₂O suggest that the aromatic rings and some methyne groups of paclitaxel are surrounded by PGDs.⁷⁵

Recently, de Groot et al. developed "cascade-release biodegradable dendrimers" that were constructed from two or more generations of branched self-elimination linkers, each of which releases spontaneously multiple leaving groups (drug) after single activation of a central core (Figure 4.13).⁷⁶ They conjugated paclitaxel via carbonate linkages with a denritic core of 4-nitrocinnamyl diol or a tetraol. The nitro function is reduced by the addition of Zn and acetic acid, which trigger spontaneous 1,8-elimination to completely release paclitaxel within 30 min. The cascade-release dendrimers, as well as the multiple release nontoxic monomers may be promising for triggered drug release, biodegradable matrices, and diagnosis.

4.6. POLYMER NETWORK AND HYDROGELS

4.6.1 NETWORK FORMATION OF MULTIFUNCTIONAL PRECURSORS

Polymer networks are generally formed using functional monomers or macromonomers by covalent and/or noncovalent bond formation. During the



FIGURE 4.12 Chemical structure of polyglycerol dendrimers with generation 3 (G-3), 4(G-4) and 5 (G-5). Reproduced from Ref. 29, with permission.



FIGURE 4.13 Concept of cascade-release dendrimers. A conjugated drug (gray) is released by single activation of the core (green). Cleavage between the core and the linker (blue) induces spontaneous self-elimination of the linkers, which lead to all the drugs rapidly. Reproduced from Ref. 76, with permission.

formation process, molecular weights of the growing precursors are increased and the crosslinking system passes through a gel point to form infinite structure. After that, the fraction of the infinite structure, *gel*, is increased at the expense of finite molecules, *sol*. The sol becomes slowly bound to the gel to form the network. High functionality (*f*: the number of functional groups in one precursor molecule) exists in dendrimers, comb-type, star-shape and hyperbranched polymers with high molecular weight as precursors of polymer network, suggesting all the functional groups are potential sites for crosslinking. In summary, the network formation shows the following features:

- 1. increasing molecular weights and molecular weight distribution;
- 2. incorporation of precursors into the network formation;
- 3. viscosity build-up before reaching gel points;
- 4. reaching gel points that are good evidence of divergence of weightaverage molecular weight; and
- 5. sol-gel transformation.

Aqueous three-dimentional networks that do not dissolve in water are called hydrogels. Hydrogels are made of hydrophilic polymers that are cross-linked by covalent bonds or physical interactions such as ionic interaction, hydrophobic interaction, and hydrogen bonding.⁷⁷ Hydrogels swells in water and, as a result, are expected to absorb at least 10 to 20% of their weight in water.

4.6.2 PHARMACEUTICAL APPLICATIONS OF HYDROGELS

Since dendrimer and hyperbranched polymers have multifunctional groups such as amino-, carboxyl-, and hydroxyl-groups, those polymers have a good potential to form a network. Crooks and Bergbreiter et al. prepared highly impermeable film by crosslinking between either amine- or hydroxyl-terminated PAMAM dendrimers and poly(maleic anhydride)-*co*poly(methyl vinyl ether).⁷⁸ When amino-terminated PAMAM dendrimers are used for crosslinking, chemical stability is increased by imidization of the maleic acid groups. Park et al. prepared hydrotropic dendrimer-based hydrogels crosslinked by ethylene glycol diglycidyl ether (EGDGE).⁷⁹ As mentioned in Section 4.5.2, the hydrotropic dendrimers solubilizes paclitaxel.²⁹ Swelling in water is increased with an increase in the concentration of EGDGE and with decreasing the concentration of dendrimers. These hydrogels become swollen in ethanol for loading paclitaxel. The hydrogels kept transparent conditions after 1 day, indicating that paclitaxel is dispersed in the hydrogels without any crystallization in water.

Hennink et al. studied protein (lysozyme, BSA, and IgG) release from dextran hydrogels physically cross-linked by stereocomplex formation of oligo(L- and D-lactide)s.⁸⁰ Controlled degradation of the hydrogels by the stereocomplex formation of comb-type oligolactide copolymers has been achieved. Especially, the release of protein with larger diameter, e.g., IgG (10.7 μ m), is governed by both Fickian diffusion and degradation. Significant properties of these proteins include quantitative release, which depends on the physicochemical characteristics of the stereocomplex. Thus, IgG release is controlled by the choice of comb-type copolymers and polydispersity of the oligo(lactide) grafts.

4.7 CONCLUDING REMARKS

Understanding polymer solution properties is essential for the design of an efficient controlled release delivery system of biologically active agents. Recent advances in syntheses of polymeric architecture using biocompatible components can provide new aspects of pharmaceutical sciences in terms of drug incorporation, solubilization, controlled release, protein stability, DNA complexation/dissociation and gene expression. Further investigation of branched polymers, polymeric micelles, dendrimers and hydrogels is believed to expand biomaterials and pharmaceutical sciences for clinical use.

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5 Protein Conjugation, Cross-Linking, and PEGylation

Kenneth D. Hinds

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5.1 INTRODUCTION

Chemical modification of proteins or other molecules with synthetic polymers often produces many benefits for their in vitro and in vivo applications. Poly(ethylene glycol) (PEG) is arguably the most extensively studied and widely used biocompatible polymer.¹ Historically, PEG polymers were used as precipitating agents for biological macromolecules (including proteins) and viruses. In addition, aqueous two-phase systems prepared with PEG, other polymers (e.g., dextran) and salts have been used to purify biological macromolecules and cells since the 1950s.² PEG is frequently used to promote cell fusion, a feature of notable importance in production of monoclonal antibodies from hybridomas.³ PEGs have been utilized for grafting onto solid supports, cross-linking of molecules (e.g., proteins and/or small molecules), linking reporter groups to biomaterials and attaching ligands to membrane-forming lipids. PEG-modified surfaces have received much attention as biomaterials because these surfaces are typically biocompatible and nonfouling (i.e., resistant to protein, bacterial, and cell adhesion). In addition, surface-bound PEGs alter the electrical nature of surfaces by forming a viscous and neutral layer that permits the control of electroosmosis in applications such as capillary electrophoresis. PEG-tethers have been employed as soluble supports in the synthesis of biopolymers (e.g., oligosaccharides, oligonucleotides, and peptides) and used as flexible linkers for drug targeting. When PEG is used as a tether for attaching molecules to surfaces, the molecules tend to function well as targeting or binding moieties. In addition, proteins attached to surfaces by PEG linkers are not denatured by interaction with the polymer-modified surface. PEG-grafted lipids and synthetic polymers have been incorporated into liposomal and micellar drug delivery systems with favorable results. PEG-based hydrogels and crosslinked networks have been utilized as biocompatible drug delivery systems, wound coverings, and cell encapsulation materials.⁴

PEG has been covalently attached (i.e., conjugated) to diverse classes of molecules for use in a variety of biological applications. PEG-modified enzymes are often soluble and capable of catalyzing chemical reactions in

organic solvents, allowing the exploitation of these conjugates in industrial settings. PEG conjugation has also been employed to improve solubility, regulate membrane permeability, and decrease the clearance of small molecule drugs. PEG-modified affinity ligands and cofactors have been used in aqueous-two phase partitioning systems for the purification and analysis of biological macromolecules and cells. PEG-saccharide conjugates are a new class of biomaterials with potential for use as drug carriers. Finally, PEG modification of oligonucleotides can improve their solubility, resistance to nucleases, and membrane permeability characteristics. While this chapter will not address specifically the subject of oligonucleotide PEGylation, an excellent review has been written on the subject.⁵

Many natural and synthetic polymers have been examined for their utility in producing pharmacologically active adducts with proteins or small molecule drugs. For example, a variety of drugs with diverse physical chemical properties have been modified using polymers such as polysaccharides, poly(amino acids), poly(vinyl alcohols), poly(vinyl pyrrolidones), poly(acryloyl morpholines), poly(acrylic acid) derivatives, polyoxazolines, and polyphosphazines.^{6–8} Although numerous substances have been modified with these different classes of polymers, this chapter will focus on the conjugation and cross-linking of proteins with PEG-based modifying reagents. The chapter begins with an overview of the properties of PEG and PEGylated proteins, followed by a survey of the protein functional groups and PEG reagents frequently exploited in conjugation and cross-linking reactions. Approaches used to prepare functional (i.e., active) conjugates; along with techniques commonly utilized to purify and characterize PEG-protein conjugates are also discussed. Finally, a brief synopsis of the relevant characteristics (e.g., purpose of modification, nature of conjugation chemistry, conjugate attributes, clinical efficacy, etc.) is provided for each of the PEG vlated therapeutic proteins currently approved for human use by the Food and Drug Administration (FDA).

5.2 PROPERTIES OF PEG AND PEG-MODIFIED PROTEINS

5.2.1 PHYSICAL, CHEMICAL, AND BIOLOGICAL PROPERTIES OF PEG

Poly(ethylene glycol) (PEG), otherwise known as poly(oxyethylene) (POE) or poly(ethylene oxide) (PEO), is a synthetic neutral polyether that is readily available in a range of sizes (i.e., molecular weights from several hundred to over ten million Daltons). These polymers are amphiphilic and soluble in water as well as many organic solvents (e.g., methylene chloride, ethanol, toluene, acetone, and chloroform). The extent of PEG's solubility in diverse classes of solvents is clearly demonstrated by the fact that concentrated solutions up to 50% have been prepared in both water and methylene chloride.⁹ In aqueous solutions PEG is a highly hydrated polymer, where each monomer (oxyethylene) unit binds two to three molecules of water due to the ether oxygen's function as a hydrogen bond acceptor.¹⁰ PEG also has a high degree of segmental flexibility and is thought to be capable of influencing the structure

of several molecular layers of water leading to its pronounced conformational entropy and large exclusion volume.^{9,11} These properties are likely the driving forces behind PEG behaving five to ten times larger than proteins of comparable molecular weight and PEG's remarkably effectiveness in excluding other polymers (both natural and synthetic) from its presence in aqueous environments. Furthermore, the exclusion of other polymers has been suggested as the reason why PEG is able to reject and precipitate proteins, is nonimmunogenic and nonantigenic, and is not degraded by mammalian enzymes.^{12–15}

Low molecular weight (<1000 Da) PEGs are viscous and colorless liquids, whereas higher molecular weight PEGs are waxy, white solids with melting points that are proportional to their molecular weights to an upper limit of about 67°C.^{1,10} PEGs are ordinarily synthesized via anionic ring opening polymerization initiated by the nucleophilic attack of hydroxide (HO⁻) ions on ethylene oxide. The molecular weight distribution (M_w/M_n), often referred to as polydispersity index, of PEG polymers is generally low (1.01 for polymers <5000 Da to 1.1 for polymers >50,000 Da) because the anionic polymerization process limits the number of chain-transfer and termination steps during reactions.¹⁶ A popular form of PEG utilized for protein modification, monomethoxypoly(ethlyene glycol) (mPEG) is prepared in the same manner as Pe.g., except methoxide anions (CH₃O⁻) are used to initiate the polymerization process resulting in polyoxyethylated species.

PEG has been found to be nontoxic and is approved by the FDA for use as excipients or as a carrier in different pharmaceutical formulations (parenterals, topicals, suppositories, nasal sprays), foods, and cosmetics.¹⁷ However, very low molecular weight (<400 Da) PEG oligomers were found to be oxidized into toxic diacid and hydroxy acid metabolites in humans via sequential oxidations by alcohol dehydrogenase and aldehyde dehydrogenase.¹⁸ The rate of PEG oxidation decreases significantly with increasing polymer molecular weight and the toxicity of larger PEGs (>1000 Da) given orally has long been known to be very low. Diets containing 16 wt% PEG (6000 Da) were found to have no adverse effects in rats.¹⁹ Extensive toxicity studies have demonstrated that PEG (4000 Da) can be safely administered intravenously as 10% (w/v) solutions to rats, guinea pigs, rabbits, and monkeys at doses up to 16 g/kg.²⁰ Finally, the subchronic toxicity of PEG (3350 Da) was studied in dogs where nine beagles were given intravenous injections of 10% polymer solutions at doses of 10, 30, or 90 mg/kg/day for 178 days, and no clinical signs of toxicity were observed.²¹

Most PEGs (>1000 Da) are rapidly removed from the body unaltered with clearance rates inversely proportional to polymer molecular weight.¹⁵ Low-molecular weight (<20,000 Da) PEGs are primarily eliminated in the urine, while larger PEGs are more slowly eliminated in both the urine and feces. After intravenous administration to humans PEG (6000 Da) was readily excreted in the urine with approximately 63% of the dose recovered after 1 h and 96%

recovered after 12 h.²² The distribution and uptake studies of radiolabeled PEGs of varying molecular weights (6000 to 190,000 Da) were examined in mice reveal the retention time of PEGs in the blood increased with increasing molecular weight and PEG's rate of urinary clearance decreased with increasing molecular weight.²³ This study also determined that PEG tended to accumulate in muscle, skin, bone, and liver to a higher extent than other organs irrespective of molecular weight, and that the rate of polymer accumulation directly correlated with its vascular permeability (i.e., decreased rates of accumulation with increasing molecular weight).

PEG is a very poor immunogen, even at extremely high (5,900,000 Da) molecular weights.⁹ Anti-PEG antibodies specific to six to seven oxyethylene units have been formed in rabbits under fairly extreme conditions where the polymer was conjugated to allergenic proteins and administered with Freund's complete adjuvant.²⁴ Fortunately, in the absence of adjuvant PEG-modified proteins do not elicit the production of anti-PEG antibodies in animals. Naturally occurring anti-PEG antibodies have been reported in only 0.2% of the normal population and no instances of anti-PEG antibody generation resulting from administration of PEG-proteins in a clinical setting have been documented.²⁵ Importantly, it is generally accepted that the minute quantities of PEG contained in therapeutic conjugates have almost no chance of eliciting unwanted host responses even with chronic use.

5.2.2 **PROPERTIES OF PEG-PROTEIN CONJUGATES**

In general, therapeutic proteins are highly potent (i.e., possess strong biological activities at very low concentrations) endogenous substances with complicated actions. These macromolecules are normally susceptible to chemical and proteolytic degradation, aggregation, polymerization, and adsorption. In addition, proteins often possess short biological half-lives, are immunogenic and antigenic, and have difficulty crossing cell membranes because of their molecular weight and solubility characteristics. Importantly, PEG conjugation can be used to increase the chemical, physical, and/or pharmacological properties of recombinant proteins because it provides a solution to many of these inherent shortcomings. Table 5.1 lists many examples of PEG-protein conjugates that have been investigated. This list is not intended to be exhaustive, rather the table serves as a resource to the reader and a connection to the primary literature where immeasurable lessons can be learned and applied to other proteins of interest.

When PEG is attached to other molecules, in general, it does not affect their chemical properties but does affect their solubility and molecular size. In addition, PEG modification of proteins commonly makes them nonimmunogenic, nonantigenic and sometimes tolerogenic; reduce the protein's rate of renal clearance and enzymatic degradation; and alter the protein's pharmacokinetics. These beneficial characteristics conferred to proteins by modification with PEG are often attributed to polymer's tendency to "mask"

TABLE 5.1Examples of PEG-protein conjugates described in the literature

Class/protein	Reference(s)
Enzymes	
Adenosine deaminase	[26-28]
Bovine liver catalase	[29, 30]
Uricase	[31–33]
Arginase	[34, 35]
Trypsin	[30, 36, 37]
Chymotrypsin	[38]
Phenylalanine ammonia lyase	[39]
Alkaline phosphatase	[40]
Substilisin	[41]
Asparaginase	$[30 \ 42-44]$
Superoxide dismutase (SOD)	[45-51]
Linase	[52-54]
Tissue plasminogen activator (TPA)	[52] 51]
I veostanhin	[55]
Panain	[50]
Streptokinase	[57]
Horseradish perovidese	[50]
Staphlokingso	[59]
Pihonuolooso A	[00]
Riboliuciease A	[50, 01]
Hematopoetic Proteins	
Granulocyte colony-stimulating factor (G-CSF)	[62–66]
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	[67–71]
Megakaryocyte growth and development factor (MGDF)	[72]
Nartograstim (NTG, G-CSF mutein)	[73]
Leridistim (II-3 and G-CSF receptor dual agonist)	[74]
Hormones and receptor antagonists	
Calcitonin	[75]
Growth hormone (GH)	[76]
Insulin	[77–84]
Epidermal growth factor (EGF)	[85]
Vapreotide	[86]
Biphalin	[87]
Growth hormone-releasing factors (GRF)	[88–90]
Growth hormone receptor antagonist	[91]
Nerve growth factor (NGF)	[92]
Octreotide	[93]
Cytokines and cytokine receptor agonists/antagonists	
Interferon- α (IFN- α -2a and IFN- α -2b)	[94–97]
Interferon- β (IFN- β -1a)	[98, 99]
Consensus interferon (IFN-con ₁)	[66]
Tumor necrosis factor-alpha (TNF- α)	[100. 101]
Interleukin-1 receptor antagonist (IL-1ra)	[68]
Interleukin-2 (IL-2)	[102–105]
	[]

Table 5.1 Continued	
Interleukin-6 (IL-6)	[106, 107]
Interleukin-8 (IL-8)	[68]
Interleukin-15 (IL-15)	[108]
Others	
Immunoglobulins (IgG)	[109, 110]
Monoclonal antibodies	[111]
Fab' fragments	[112–115]
Single chain (scFv) antibodies	[116]
Antisense oligonulceotides (anti-ODN)	[5]
Cyanovirin-N	[117]
Brain-derived neurotrophic factor (BDNF)	[118]
Type 1 TNF α soluble receptor	[119, 120]
Hemoglobin	[121-125]
Hirudin	[126]
BSA	[14]
Ovalbumin	[127, 128]
Cytochrome P450 (1A2)	[129]
A2-macroglobulin	[46]
Collagen	[130]
Aptamers	[131]
Viruses	[132–134]

specific protein domains from recognition by other macromolecules such as enzymes, antibodies, and receptors.

It is widely accepted that most parenterally administered proteins are rapidly cleared from the body by the reticuloendothelial system (RES), kidney, spleen, and liver. Moreover, clearance depends on molecular size, charge, and the presence of specific cellular receptors for the proteins of interest. PEG modification naturally increases a protein's molecular size, often alters molecular charge, and ordinarily diminishes receptor-binding capabilities, leading to a reduction in the modified protein's rate of clearance. Furthermore, the enzymatic degradation of therapeutic proteins is accompanied by a rapid decline in biological activity and therapeutic efficacy. By sterically shielding the protein domains susceptible to proteolytic attack, PEG can decrease the amount of protein that is degraded and rendered biologically inactive. Even recombinant human proteins are known to elicit immune responses after repeated use. By sterically masking the therapeutic protein's immunogenic/ antigenic determinants, PEG attachment commonly produces conjugates that are nonimmunogenic and nonantigenic. This aspect of PEGylation is especially beneficial for increasing the therapeutic efficacy of heterologous proteins because (the frequently encountered) circulating antibodies may neutralize the native protein's biological activity (typically IgG subclass) or induce lifethreatening hypersensitivity reactions (IgE subclass). PEGylation can also prevent the nonspecific adsorption of opsonins (a poorly characterized class of serum proteins that includes albumin and IgG antibodies) to a protein's surface, enabling the conjugate to avoid the opsonization process (receptormediated recognition and removal of protein–opsonin complexes by phagocytes/macrophages) and clearance by RES tissues.¹³⁵ Because of the reasons mentioned above, it should be readily apparent that protein modification with PEG has the potential to create clinically relevant medicines in cases where the unmodified protein's properties are not suitable for use as therapeutics.

5.3 CHEMISTRY OF PROTEIN FUNCTIONAL GROUPS

5.3.1 COMPOSITIONS OF PROTEINS

All proteins and peptides are polymers of amino acids. There are twenty naturally occurring amino acids that can be sub-divided into four basic groups depending on the size, shape, charge, and relative hydrophobicity or hydrophilicity of their side-chains. Residues such as glycine (G, gly), alanine (A, ala), valine (V, val), leucine (L, leu), isoleucine (I, iso), proline (P, pro), and methionine (M, met) have nonpolar aliphatic side chains while phenylalanine (F, phe) and tryptophan (W, trp) have nonpolar aromatic side chains. Residues such as arginine (R, arg), glutamic acid (E, glu), aspartic acid (D, asp), cysteine, (C, cys), histidine (H, his), lysine (K, lys), and tyrosine (Y, tyr) have ionizable polar side chains while asparagine (N, asn), glutamine (Q, gln), serine (S, ser), and threonine (T, thr) contain non-ionizable side chains. Nonpolar residues are generally found within the interior (hydrophobic core) of globular proteins or are otherwise protected from aqueous environments by various folding motifs while amino acids with polar side chains are commonly located in solvent accessible areas of proteins where they can interact with the aqueous environment. As a general rule, a protein's amino acid composition (primary sequence), secondary, tertiary, and even its quaternary structure will dictate the accessibility and reactivity of its functional groups towards modifying agents like Pe.g.

5.3.2 REACTIVITY OF PROTEIN FUNCTIONAL GROUPS

As mentioned above, the chemical reactivities of proteins depend on the sidechains of their constituent amino acids, as well as their free amino and carboxyl groups of the N- and C-terminal residues. The alkyl side-chains of the hydrophobic amino acids and the aliphatic hydroxyls of serine and threonine are, for all conjugation purposes, chemically inert. Furthermore, methionine and tryptophan residues are ordinarily buried within the interior of proteins and because of their limited reactivity based on steric constraints, are not considered ideal targets for modification. Oppositely, the ionizable groups mentioned previously are usually located at (or near) the surface of proteins and therefore are the normal targets of protein cross-linking and conjugation with PEG.¹³⁶ Table 5.2 provides the structures and properties of the chemically reactive amino acid (side-chains) that are potentially favorable sites for chemical modification with mPEG or cross-linking with PEG.

Amino acid	Chemical Structure	pK _a ¹	pK _b ²	рК _х ³	pl ⁴
Glutamic acid (Glu, E)	ОН О=С О СН-СН ₂ -С-ОН И NH ₂	2.19	9.67	4.25	3.22
Aspartic acid (Asp, D)	ОН О=С О СH-СH ₂ -СH ₂ -С-ОН I NH ₂	1.88	9.60	3.65	2.77
Cysteine (Cys, C)	OH O=C CH-CH ₂ -SH NH ₂	1.96	10.28	8.18	5.07
Lysine (Lys, K)	$\begin{array}{c} OH \\ O=C \\ C \\ CH-CH_2 - CH_2 - CH_2 - CH_2 - NH_2 \\ NH_2 \end{array}$	2.18	8.95	10.53	9.74
Arginine (Arg, R)	$\begin{array}{c} OH \\ O = C \\ I \\ C H - C H_2 - C H_2 - C H_2 - NH - C - NH_2 \\ I \\ N H_2 \end{array}$	2.17	9.04	12.48	10.76
Serine (Ser, S)	ОН О=С СН-СН ₂ -ОН I NH ₂	2.21	9.15	N.A.	5.68
Tyrosine (Tyr, Y)	OH O=C CH-CH ₂ -OH NH ₂ -OH	2.20	9.11	10.07	5.66
Histidine (His, H)		1.82	9.17	6.00	7.59

TABLE 5.2 Properties of the amino acids commonly targeted for modification

¹ Negative logarithm of the dissociation constant for the $-CO_2^-$ group. ² Negative logarithm of the dissociation constant for the $-NH_3^+$ group. ³ Negative logarithm of the dissociation constant for the side chain functional group. ⁴ pI is the pH at the isoelectric point for the specified amino acid.

The relationship between nucleophilicity and reactivity must be addressed when considering which group(s) of a protein to target for PEGylation because most conjugation reactions are nucleophilic reactions involving the displacement of a leaving group by an attacking nucleophile. Furthermore, alkylation (an alkyl group is bonded to the nucleophilic atom) and acylation (an acyl group is bonded to the nucleophilic atom) reactions are the most important used in protein modification with PEG The rates of such bimolecular nucleophilic, $S_N 2$, reactions are largely dependent on two factors: (1) the ability of the leaving group to leave; 137 and (2) the nucleophilicity of the attacking group.¹³⁸ In the most frequently employed protein modification schemes, where electrophilically activated PEGs are used to target protein nucleophiles, the reaction rate is essentially a function of the nucleophilicity of the different amino acid side chains because the polymer's leaving group does not vary. In this chapter a nucleophile is defined as any species (neutral or negative) that has an unshared pair of electrons, i.e., a Lewis base. Because protonation effectively decreases the nucleophilicity of a species, the selection and control of the pH in a reaction system is of prime importance and substantially affects the rates of nucleophilic reactions. According to theory, the relative order of nucleophilicity of the major reactive groups in biological molecules can be summarized as follows:

 $R-S^{-} > R-SH$ $R-NH_{2} > R-NH_{3}^{+}$ $R-CO_{2}^{-} > R-CO_{2}H$ $R-O^{-} > R-OH$ R-OH = H-OH

and finally:

$$R-S^- > R-NH_2 > R-CO_2^- = R-O^-$$

Of course, the relationship between pH and the degree of protonation of a nucleophile depends on its pK_a according to the commonly encountered Henderson–Hasselbach equation:

$$pH = pK_a + \log([base]/[acid])$$

Using these general relationships, the strongest nucleophile in a protein will be the ionized thiolate group of cysteine. The next most reactive protein nucleophiles are the unprotonated amines, including the primary amino groups at the N-termini and the ε -amino groups of lysine side chains; the secondary amines of histidine imidazolyl and tryptophan indolyl side chains; and the guanidino amines of arginine side chains. Finally, the least reactive protein nucleophiles are the ionized forms of oxygen-containing groups, including the α -carboxyl groups at the C-termini, the β -carboxyl groups of aspartic acid side chains, and the γ -carboxyl groups of glutamic acid side chains; the carbinol groups of serine side chains; and the phenolate groups of tyrosine side chains.

The relative reactivity of a nucleophile is influenced by specific factors such as solvation, size, and bond strength; or by more general environmental factors like the reaction medium's temperature and ionic strength.¹³⁶ In addition, the unique microenvironment of individual protein nucleophiles can substantially affect their corresponding pK_a values. For example, the pK_a values for ionizable protein nucleophiles can be shifted by perturbations that are caused by neighboring charged groups, which stabilize ionic species of opposite charge and destabilize those of the same charge. Moreover, the partial burying or shielding from aqueous solvent can not only provide a kinetic barrier to approaching modifying reagents, but also destabilizes ionized species of either charge. Thus, many environmental variables can be manipulated and optimized in order to modify of only those particular residues necessary for the production of biologically active PEG-protein conjugates. Unfortunately, the selective modification of proteins with PEG is not a trivial exercise and therefore the selection of rational synthetic schemes utilizing the appropriate activated PEG and reaction conditions is essential.

5.4 PEG REAGENTS FOR CONJUGATION OR CROSS-LINKING

Poly(ethylene glycol) polymers are especially useful protein conjugation and cross-linking reagents because they are available in a wide variety of molecular weights, and numerous functionalized and backbone architectures. The molecular weights of linear PEGs used in biomedical applications usually range between a few hundred to 20,000 Da, but polymers up to 50,000 Da have also been reported. In addition to linear forms of PEG, polymers with branched, forked, branched-forked, and multi-arm configurations have also been used to modify proteins. Diverse classes of reactive functional groups have been introduced to PEG polymers using chemical methods that transform of their (normally) unreactive hydroxyl(s). Advances in PEG chemistry have produced polymers that reversibly modify proteins allowing for the release of a protein in its native form. The following sections are intended to illustrate the numerous classes of reagents that can be used in protein modification schemes, along with representative reaction conditions, and the potential advantages and disadvantages of using each. This section is far from comprehensive, but should provide a glimpse of the tremendous flexibility afforded scientists attempting to design PEG polymers and/or approaches to effective protein modification. Some factors to take into account when deciding which type of modifying reagent should be used to include polymer size and shape, identity of functional groups(s), degree of selectivity required, potential impact of leaving groups on conjugate properties, and desired stability of the linkage between polymer and protein.

5.4.1 **PEG STRUCTURES**

Linear PEGs are available in difunctional and monofunctional forms permitting telechelic (PEGs designed to contain terminal functional groups at both ends) and semitelechelic (PEGs designed to contain only one terminal functional group) species to be prepared, respectively.¹³⁹ Linear telechelic PEGs can be further categorized, according to the likeness of introduced terminal functional groups, as homo-bifunctional (identical) or heterobifunctional (different) species. Homobifunctional PEGs are regularly used as water soluble, flexible, and biocompatible macromolecular cross-linking agents, while heterobifunctional PEGs are frequently used to join certain functional molecules to other materials (e.g., surfaces, synthetic polymers, biopolymers, liposomes, etc.). Linear semitelechelic forms of PEG (i.e., activated mPEGs) are normally used in protein conjugation reactions because of their ability to minimize unwanted cross-linked products. However, the mPEGs obtained from commercial sources can have levels of PEG-diol impurities sometimes exceeding 15%.¹⁴⁰ The removal of these impurities prior to polymer activation and protein conjugation is extremely important if relatively homogeneous conjugates are desired.

In addition to the linear forms of PEG and mPEG mentioned above, numerous other backbone architectures have been used to modify proteins. Figure 5.1 illustrates many of the backbone architectures utilized to modify proteins. The first branched PEG derivative was prepared by attaching two mPEG chains to 2,4,6-trichloro-s-triazine (cyanuric chloride), leaving the remaining chlorine group available for protein modification.¹⁴¹ This reagent was less than useful due to its low reactivity towards protein amino groups. Another branched PEG polymer form was prepared by attaching two chains of linear mPEG to the α - and ε -amino groups of lysine followed by conversion of the lysine carboxylic acid to an activated NHS-ester.¹⁴² This branched reagent was found to be far more useful due to its high reactivity and the fact that the lysine carboxylate can be easily converted to any number of different functional groups (e.g., aldehyde, thiol, maleimide, etc.) using chemical methods.¹⁶ Branched mPEGs of this sort are sterically bulky, high molecular weight (up to 60,000 Da), and monofunctional compounds that permit the attachment of two polymer chains to a protein by way of a single point of attachment.³⁰ Importantly, when branched mPEGs are attached to proteins they behave larger, are more effective in reducing antigenicity and immunogenicity, and are more effective in protecting proteins from proteolysis than linear mPEGs of the same molecular weight.^{30,143}

More recently, forked backbone architectures comprised of a single PEG chain with two reactive groups in close proximity to each other have been



FIGURE 5.1 Various PEG backbone architectures. (A) Branched (PEG2), (B) linear forked PEG, (C), branched-forked PEG, (D) multi-arm PEG, and (E) pendant PEG [Y is a group with a carbon branching moiety and X is a molecule, linker and/or functional group]. Adapted from Roberts et al., *Adv. Drug Del. Rev.*, 2002; **54**: 459.

prepared by attaching a trifunctional linker (e.g., serinol or β -glutamic acid) to mPEG.¹⁴⁴ This structural form of polymer might be used to mimic the heavy chain domains of antibodies by conjugating two F(ab)' fragments to the same end of the polymer.¹⁶ In addition, branched-forked forms of PEG having two mPEG chains attached to a single core lysine and two reactive groups in close proximity to each other are available for applications where a more sterically "bulky" forked PEG is advantageous for protein modification (Nektar Molecule Engineering Catalog, 2003). Pendant-modified PEG NHS-esters have been prepared by alternating copolymerization of lysine with PEG macromers, where the lysine carboxylate side chains are evenly distributed along a linear backbone and available for protein modification following activation.¹⁴⁵ Finally, multi-arm (3–8 arm) backbone architectures also have been prepared via ethoxylation of polyols and these branched reagents may be functionalized for protein modification.

5.4.2 FUNCTIONALIZED PEGS

Although, in some instances the hydroxyl end-groups of PEG or mPEG polymers can be directly used to modify proteins, the majority of cases require some sort of polymer activation prior to conjugation or cross-linking. A large number of chemical methods have been used to introduce reactive functional groups to PEG and mPEG polymers through their terminal hydroxyl group(s). Such activated PEGs are designed to selectively react with complementary functional groups found in proteins and the linkages between polymer and protein can be permanent or reversible. It should be noted that many of the early examples of semitelechelic mPEG reagents suffered from high levels of difunctional and unreacted polymeric impurities. This is because they were prepared from starting materials that contained appreciable amounts of diol-PEG by the direct transformation of the polymer's hydroxyl group to a unionized reactive functionality, making the removal of such polymeric impurities that had similar physicochemical properties extremely difficult. However, the availability of very high purity mPEGs and the use of activation protocols that provide ionizable intermediates capable of being purified using ionexchange chromatography have dramatically changed the current situation.

5.4.2.1 Nucleophilic PEGs

5.4.2.1.1 PEGs for Carboxyl Modification

Some of the earliest reported PEGylation reactions involved the attachment of nucleophilic PEG derivatives to activated carboxyl groups of proteins. PEGamine¹⁴⁶ and PEG-*p*-aminobenzylether¹⁴⁷ derivatives have both been attached to carbodiimide-activated carboxylic groups of proteins. Unfortunately, when PEG-amine is used the selectivity of these reactions is rather poor because of the similar reactivities of PEG-amine and the amino groups of the protein. This problem can be circumvented to a certain extent by using PEG-paminobenzylethers because the pK_a of the aromatic amino group is lower than the primary amino groups of the protein. Thus, when a slightly acidic pH (4.8–6.0) is selected for the conjugation reaction the polymer is expected to preferentially react with carbodiimide-activated carboxyl groups of a protein. Otherwise, one can (reversibly) protect the protein's amino groups prior to modification with a nucleophilic PEG species and later remove (i.e., deprotect) the protecting groups from the PEG-protein. However, when using carbodiimide-mediated coupling of a nucleophilic PEG to a protein there is the possibility of side reactions such as modification of tyrosyl and cysteinyl side groups and the formation of N-acylurea derivatives.^{148,149} PEG-hydrazide has also been used to specifically modify the carbodiimide-activated carboxyl groups of proteins.¹⁵⁰ This method takes advantage of the fact that the hydrazide group has a very low pK_a ($pKa \sim 3$) and is capable of reacting under acidic conditions (pH 4.5-5) where protein amino groups are protonated and unreactive.

5.4.2.1.2 PEGs for Oxidized Carbohydrates or N-terminus Modification

Vicinal diols commonly found in the carbohydrate groups of glycoproteins can be oxidized enzymatically (e.g., glucose oxidase) or chemically (e.g., sodium periodate) to supply a specific site for protein modification using nucleophilic PEGs. However, the same problems observed when protein carboxylate groups are targeted for amidation are also encountered in reactions involving the reductive amination of aldehydes. Thus, using PEG-hydrazide, PEG-*p*aminobenzylether, or protection/deprotection strategies will bolster the chances of specifically modifying the targeted aldehyde groups. When PEGhydrazide is reacted with the aldehyde group(s) contained in glycoproteins a hydrazone bond is formed between the polymer and protein that can be reduced using sodium cyanoborohydride to the corresponding alkyl hydrazide. When the PEG-amines are reacted with the aldehyde group(s) contained in glycoproteins the reaction proceeds through the formation of a reversible Schiff-base that can also be reduced using sodium cyanoborohydride to the corresponding secondary amine.

A very interesting and highly selective PEGylation strategy was developed that relies on the ability to generate a reactive carbonyl group in the place of the terminal amino group of proteins.⁶⁸ If the protein has an N-terminal serine or threonine residue, then mild periodate oxidation can generate a glyoxylyl group suitable for conjugation using aminooxy-PEG. The result of the modification reaction is a hydrolytically stable oxime bond between the polymer and protein. A method less restricted by the nature of the N-terminal residue was also disclosed that also utilizes aminooxy-PEG and N-terminal oxidation, but requires harsher metal-catalyzed transamination to provide a reactive keto group for conjugation. Conjugates produced using this N-terminal oxidation approach are more likely to retain their biological activities since only a single polymer chain is placed at a very specific position. Reaction schemes along with the products resulting from using these conjugation approaches are given in Figure 5.2.

5.4.2.2 Electrophilic PEGs

5.4.2.2.1 Acylating PEGs for Amine Modification

The vast majority of early research in the field of PEGylation centered-around acylating the lysine side chains of proteins with electrophilically activated polymer derivatives. This focus was brought about because of three good reasons: (1) lysine residues are relatively abundant in proteins, (2) lysines are commonly located near the surface of proteins, and (3) the amino groups of lysine side chains are potent nucleophiles. Figure 5.3 shows the chemical structures and reaction pathways for some of the first-generation acylating reagents described below.

(A)



FIGURE 5.2 PEG modification of introduced reactive carbonyl groups. (A) oxidized carbohydrates of glycoproteins and (B) oxidized N-terminus. Adapted from Roberts et al., *Adv. Drug Del. Rev.*, 2002; 54: 459.

PEG-succinimidyl succinate (SS-PEG) has also been prepared and used to modify therapeutic proteins via reaction with their amino groups.⁴³ This form of activated PEG is usually prepared by succinylation of the terminal hydroxyl group(s) of PEG followed by carbodiimide-mediated condensation with N-hydroxy-succinimide (NHS). This NHS-activated PEG-succinimide can react with proteins within a short time period under relatively mild conditions (30 min, pH < 7.8, 25°C) to produce conjugates with well-preserved biological activites.^{41,43} Unfortunately the ester linkage between the polymer and the succinic ester group is susceptible to hydrolysis, and the conjugates prepared by this method slowly lose their desired properties due to the hydrolysis of the PEG chains. The rates of hydrolysis can be slowed slightly by substituting the succinate group with a glutarate (SG-PEG)¹⁰² and substantially slowed by replacing the aliphatic ester with an amide bond (SSA-PEG).⁴³

PEG-imidazole carbonate derivatives have been prepared by reaction with either imidazolyl formate ¹⁵¹ or carbonyldiimidazole.¹⁵² These derivatives couple to protein amino groups through a urethane linkage under mild conditions.⁴⁶ This activated form of PEG is significantly less reactive than the succinimidyl esters and may require extended (48–72 h, pH 8.5) reaction times. The slower reaction rate can make the reagent more selective and able to



FIGURE 5.3 First-generation acylating PEG derivatives for amine modification. Adapted from Roberts et al., *Adv. Drug Del. Rev.*, 2002; 54: 459.

selectively modify certain amino groups in proteins, producing conjugates with preserved biological activity.¹⁶ However, this reagent is not the best choice for modifying unstable molecules, as the long times required for reaction can cause irreversibly degrade the molecule's activity.

PEG-phenylcarbonates have also been used to produce conjugates via the formation of a carbamate bond between the activated polymer and the amino group(s) of a protein.¹⁵³ The advantages of this method are that it utilizes commercially available chloroformates of 4-nitrophenol (NPC-PEG)

or 2,4,5-trichlorophenol (TCP-PEG) and a single-step activation protocol. These reagents are somewhat more reactive than the PEG-oxycarbonylimidazole derivative mentioned above allowing for shorter reaction times (24 h) under the same (pH 8–8.5) mild aqueous conditions. Modification reactions using NPC-PEG can be followed spectrophotometrically ($\varepsilon_{400 \text{ nm}} = 17,000 \text{ M}^{-1}$ cm⁻¹ for *p*-nitrophenol in basic solution). In addition, using TCP-PEG for protein modification permits monitoring of the reaction progress using the Habeeb method, as the carbonate does not interfere with the TNBS assay of free amino groups. Unfortunate disadvantages of this method are that the reaction by-products (4-nitrophenol and 2,4,5-trichlorophenol) are toxic, hydrophobic, and may have nonspecific affinities toward some proteins.⁹

PEG-succinimidyl carbonate (SC-PEG) is another species of activated polymer that specifically reacts with a protein's amino groups via a carbamate linkage.¹⁵⁴ SC-PEGs can be prepared by a one-pot synthetic method, and they have been found to be even more selective than SS-PEGs for reaction with the amino groups of proteins. In addition, relatively short reaction times (30 min) over a broad pH range (maximum reactivity at pH 9.3), and the fact that the liberated NHS is nontoxic and unreactive towards proteins makes this a nearly ideal method for producing a minimally toxic conjugate with preserved biological activity. PEG-benzotriazole carbonate (BTC-PEG) is a similar activated polymer that selectively modifies the amino groups of proteins by forming urethane (carbamate) bonds.¹⁵⁵ As with SC-PEG, the modification of protein amino groups with BTC-PEG is normally accomplished under mild conditions within short periods of time (e.g., pH 8.5 aqueous buffer, 25°C, 30 min). The major difference between SC-PEG and BTC-PEG is that the latter is less stable to hydrolysis, and therefore somewhat less selective. Both SC-PEG and BTC-PEG are more selective than SS-PEGs in reacting with protein amino groups and are capable of producing extensively modified proteins with well-preserved activity. As recent reports have indicated and depicted in Figure 5.4, both SC-PEG and BTC-PEG may react with histidine and tyrosine residues forming hydrolytically unstable imidazolecarbamate⁹⁶ and phenylcarbonate ¹⁵⁶ linkages, respectively. Such unexpected side reactions most likely occur due to the unique microenvironmental conditions that accentuate the nucleophilicity of the particular side chains involved. However, these types of hydrolytically unstable linkages between polymer and proteins may be capitalized on for creating pro-drug strategies when PEGylation is detrimental to protein activity.

PEG-xanthates (dithiocarbonate) have also been used to covalently modify the amino groups of proteins via formation of a thionourethane linkage.^{157,158} This approach has the distinct advantage of providing a method of conveniently determining the composition of PEG-oxythiocarbonylated proteins spectrophotometrically from an increase in the ultraviolet absorption at 242 nm as compared to the native protein. Additionally, the conjugates showed good chemical stability in various buffer systems the reactions can be carried out under relatively mild conditions in the pH range of 9 to 10. PEGisocyanate (PEG-NCO) is another useful, yet unselective reagent used to



FIGURE 5.4 Atypical modification of histidine or tyrosine residues with SC- or BTC-PEG. Adapted from Veronese et al., *Biomaterials*, 2001; 22: 405.

modify hydroxyl and amino groups.^{149,159} In aqueous medium at a basic pH this polymeric reagent can react with protein hydroxyl and amino groups forming urethane and urea bonds, respectively.

The NHS esters of PEG-carboxylic acids are now the most popular derivatives for PEGylating proteins and reaction schemes for this class of reagents are given in Figure 5.5. The advantages of using this class of polymers are their backbone, which contains only ether bonds and an amide linkage, making them useful for preparing stable conjugates. The succinimidyl ester of carboxymethylated PEG (SCM-PEG) was the first PEG-carboxylate derivative used for protein modification.¹⁶⁰ SCM-PEG is extremely reactive towards both hydrolysis ($t_{1/2} \approx 1 \text{ min at pH 7.5, } 27^{\circ}\text{C}$) and aminolysis, limiting the reagent's selectivity and increasing the probability of unwanted side reactions. The cause



FIGURE 5.5 PEG NHS esters. (A) PEG NHS esters based on propionic and butanoic acids and (B) α -branched PEG NHS esters based on propionic and butanoic acids. Adapted from Roberts et al., *Adv. Drug Del. Rev.*, 2002; **54**: 459.

of SCM-PEG's heightened reactivity was hypothesized to be the close proximity (one methylene group spacer) of the electron-withdrawing ether oxygen and the electrophilic carbonyl group of the NHS ester. Two less reactive PEG-carboxylate NHS ester reagents were created by elongating the spacer between the ether oxygen and the carbonyl group. To this end, SPA-PEG (NHS ester of PEG propionic acid) and SBA-PEG (NHS ester of PEG butanoic acid) derivatives were prepared with two and three carbon spacers, respectively. As seen in Table 5.3, and substantiating the hypothesis outlined above, SPA-PEG ($t_{1/2} \approx 16.5$ min at pH 8, 25°C) is in fact slightly more reactive than SBA-PEG ($t_{1/2} \approx 23.3 \text{ min}$ at pH 8, 25°C) and these reagents are substantially more stable than SCM-PEG. The reactivity of the NHS-activated PEG-carboxylic acids can be further decreased by introducing an α -branching moiety to the carboxylic acid, and the α -methyl branched derivative of SPA-PEG has an even longer hydrolysis half-life ($t_{1/2} \approx 33 \text{ min}$ at pH 8, 25°C) than SBA-PEG Importantly, both SPA-PEG and SBA-PEG have been successfully used to modify proteins in a selective manner by modulating reaction conditions. Finally, certain conjugates that have progressed to late stage (i.e., Phase III) clinical trials were prepared using both SPA-PEG and SBA-PEG, further establishing their utility.

5.4.2.2.2 Alkylating PEGs for Amine Modification

In addition to the first-generation acylating reagents, many types of early PEGbased alkylating reagents were prepared and tested. Figure 5.6 shows the structures of a few of these alkylating reagents and the nature of the bond formed between the polymer and protein when such reagents are used. A oncepopular method for covalently attaching PEG to protein via their amino group(s) was to react the primary alcohol group of PEG with one of the

TABLE 5.3

Comparison of reactivity of various electrophilically activated PEG-ester derivatives measured by hydrolysis half-lives^a at pH 8, 25° C

PEG-NHS Ester Symbol		Half-life (minutes) ^b		
mPEG-O-CH ₂ CH ₂ CH ₂ -CO ₂ -NHS	SBA-PEG	23.3		
mPEG-O-CO ₂ -NHS	SC-PEG	20.4		
mPEG-O ₂ C-CH ₂ CH ₂ CH ₂ -CO ₂ -NHS	SG-PEG	17.6		
mPEG-O-CH2CH2-CO2-NHS	SPA-PEG	16.5		
mPEG-O-CO ₂ -BTC	BTC-PEG	13.5		
mPEG-S-CH ₂ CH ₂ -CO ₂ -NHS	SSPA-PEG	10.7		
mPEG-O ₂ C-CH ₂ CH ₂ -CO ₂ -NHS	SS-PEG	9.8		
mPEG-O ₂ CNH-CH(Bu)-CO ₂ -NHS	NOR-PEG	5.4		
mPEG-O2-CNH-CH(R)c-CO2-NHS	mPEG2	4.9		
mPEG-NH-CO-CH2CH2-CO2-NHS	SSA-PEG	3.2		
mPEG-O-CH2-CO2-NHS	SCM-PEG	0.75		

^aAminolysis rates parallel hydrolysis rates. ^bTypically, the hydrolysis half-lives triple upon lowering pH one unit. ^c $R = -(CH_2)_4$ -NH-CO₂-(mPEG). (From Shearwater Polymers Catalog, 2000.)



FIGURE 5.6 Alkylating PEG derivatives and PEG-imidoesters for amine modification.

chlorine groups of trichloro-s-triazine (cyanuric chloride), leaving the two remaining chlorine groups available for protein modification.¹⁴ Although this approach provides a very effective way of preparing an activated polymer for conjugation to proteins, it also suffers from many disadvantages such as toxicity of the cyanuric chloride and the excessive reactivity of the PEG-triazine towards nucleophiles other than amines (cysteinyl thiols and tyrosyl hydroxyls). Furthermore, cyanuric chloride-activated PEG is considered to be the least suitable reagent for the selective modification of proteins, and modification of some proteins with this form of activated PEG often results in conjugates with greatly diminished biological activities.⁹

PEG-tresylates are sufficiently reactive towards protein amino groups at near-neutral conditions, and the bond that results from this conjugation method is a very stable secondary amine that does not change the total charge of the conjugate relative to the native protein.¹⁶¹ In addition, the protein conjugates can be conveniently characterized by quantitation of lysines by amino acid analysis or fluorescamine assay (specific to primary amino groups).^{162,163}

PEG-imidoesters have also been used to modify proteins through reaction with their aminogroups.¹⁶⁴ These activated polymers react with protein amino groups at pH 7–9 resulting in an imidyl linkage. One advantage of modifying a protein using this type of polymer is the aminidated protein possesses the same net charge as the unmodified species, which can be important if the target group's positive charge is essential for activity. In addition, the imidyl bond formed between the polymer and protein is hydrolytically stable, and survives the extreme conditions required for amino acid analysis allowing the quantification of a conjugate's degree of substitution by diminished lysine content.⁹

PEG-glycidyl ether (PEG-epoxide) is a mildly reactive electrophilic reagent that is coupled to protein nucleophiles under relatively basic (pH 8.5–9.5) conditions.¹⁶⁵ This form of activated polymer alkylates protein amino, hydroxyl, and thiol groups, resulting in secondary amine, alkyl ether and thioether linkages. Although the epoxide is significantly less reactive than PEG NHS esters, it is very stable to hydrolysis in basic conditions and the conjugation reaction does not produce a leaving group. Under normal physiological conditions, the overall molecular charge of conjugates produced using this reagent will be unchanged relative to the native protein.

PEG-aldehydes are extremely useful modifying reagents because they specifically react with protein amino groups without interference from other protein' nucleophiles.¹⁶⁶ Figure 5.7 shows the coupling reaction of PEG-aldehyde to protein amines, which proceeds through a reversible Schiff base intermediate that is subsequently reduced (typically with sodium cyanobor-ohyride or sodium borohyride) forming a stable secondary amine linkage between the polymer and protein. A further useful property of this approach to protein modification is the ability to achieve some selectivity between the N-terminal α -amine(s) and any lysine ε -amine(s) contained in proteins by employing mildly acidic reaction conditions (pH ~ 5–6) that favor reaction





TABLE 5.4 Comparison of reactivity of various PEG derivatives toward protein lysine (ε -amino) groups

PEG Derivative	Lysine reactivity relative to SS- or SPA-PEG (pH 8–9)			
	Similar	Faster	Slower ^b	Much slower ^c
CM-PEG		X ^a		
NOR-PEG		\mathbf{X}^{d}		
SC-PEG	Х			
Tresyl-PEG			Х	
Aldehyde-PEG				Х
Epoxide-PEG				Х
Carbonylimidazole				Х
PNP-carbonate PEG			Х	
^a Reaction over in 1–2 minut	es.			
^b Try 1–2 hour reaction time.				
Try 18–24 hour reaction tin	ne.			
"Try 5–10 minute reaction ti	me.			
(From Shearwater Polymers	Catalog, 2000).)		

with the α -amino group due to its lower pK_a . Although complete selectivity is not achieved, the conjugates prepared in this manner are far less heterogeneous than those prepared using other electrophilic PEG reagents. Numerous examples of selective α -amine modification of proteins and peptides will be given in the next section. Finally, Table 5.4 compares the relative reactivity of several classes of PEG derivatives towards protein lysine (ε -amino) groups.

5.4.2.3 PEGs for Cysteine Modification

Figure 5.8 illustrates many of the useful PEG reagents that been prepared to selectively modify cysteine thiol groups. PEG-maleimides (MAL-PEG) reagents have been prepared for selective cysteine modification.^{167,168} The reaction of the PEG-maleimide with protein free-thiol groups occurs via Michael-addition under mild conditions (pH 6–7) forming a hydrolytically



FIGURE 5.8 Thiol reactive PEGs. (A) PEG-maleimide, (B) PEG-vinylsulfone, (C) PEG-iodoacetamide, and (D) PEG-orthopyridyl disulfide. Adapted from Roberts et al., *Adv. Drug Del. Rev.*, 2002; **54**: 459.

stable thioether linkage between polymer and protein, and this reaction can be selective even in the presence of other nucleophilic functional groups. However, the maleimide is known to undergo slow addition of water across the double bond and ring opening amide bond hydrolysis in a conjugate may also occur.¹⁶⁹ PEG-vinyl sulfone (VS-PEG) is a derivative that also selectively modifies the free (cysteine) sulfhydryl groups of proteins.¹⁷⁰ For example, under near neutral conditions (e.g., pH 7-8) VS-PEG will react with protein thiols with a half-life around 15-20 min resulting in a stable thioether bond between polymer and protein, but the reaction will proceed faster at higher pH. However, the vinylsulfone group is a little less reactive than the maleimide. It has problems reacting with sterically crowded sulfhydryl groups, and under certain conditions (e.g., pH 9-9.5, long reaction time). The VS-PEG can also undergo Michael-addition reactions with primary amines. The major advantage of using VS-PEG over MAL-PEG is that the former is stable to hydrolysis and a stable linkage is formed between the polymer and protein. PEG-orthopyridyl-disulfide (OPSS-PEG) efficiently reacts with protein sulfhydryl groups under both acidic and basic conditions to form a disulfide bond that is readily cleaved to regenerate the native protein under mildly reducing conditions.⁵⁷ Reportedly, the PEG-S-S-Protein disulfides produced in this way are stable under physiological conditions, except in a reducing environment like that found in the lysosome.¹⁶ PEG-iodoacetamide is a less frequently used sulfhydryl selective reagent that reacts slowly with protein thiols by nucleophilic substitution via formation of a thioether bond.¹⁶⁸ It is important to note that modification reactions using this reagent should be kept in the dark to prevent any liberated free iodine from reacting with other amino acids (i.e., tyrosine). Finally, readers may notice that thiol-PEG (SH-PEG) reagents were not discussed in this section. This is due to the fact that the thiol is a nucleophilic derivative that readily undergoes disulfide formation/exchange, acylation, and alkylation reactions with a variety of amino acids, and thus is in no way considered selective for cysteine modification.

5.4.2.4. PEGs for Introduction of Reporter Groups

Amino acid esters of PEG have terminal primary amino groups available for modification of proteins or glycoproteins using the same approaches mentioned above.¹⁷¹ These derivatives are prepared in such a way that there is an aliphatic ester between the polymer and amino acid that can be cleaved by hydrolysis or hydrazinolysis leaving a reporter group to aid in the characterization of conjugates derived from them by amino acid analysis. In addition, the lower pK_a of this reagent's α -amino group ($pK_a \sim 7-8$) relative to a simple PEG-amine ($pK_a \sim 10$) or a protein's ε -amino groups allows for more selective modification of protein electrophiles under neutral or slightly acidic conditions.

PEG-amino acid NHS esters have also been used to introduce reporter groups to proteins in a manner similar to that of nucleophilic PEG amino acids mentioned above. These reagents have been prepared by reacting PEGphenylcarbonates with an appropriate amino acid, followed by conversion of the carboxylate group to an activated NHS ester.³⁵ This class of reagents is extremely versatile and can greatly aid in the analysis of PEG-proteins because the carbamate-bond between polymer and amino acid is labile in the extreme acid hydrolysis conditions (6 N HCl, 100°C, 12-24h) used in amino acid analysis. For example, the amino acid spacer may contain an unnatural amino acid (i.e., norleucine) that is easily detected by amino acid analysis permitting the convenient and direct determination of the number of polymer chains attached to a protein. In addition, commercially available radioactive amino acids can be incorporated into the spacer for conjugate detection in pharmacokinetic or metabolic studies. It is also possible to monitor the microenvironment of the water-polymer-protein interface using a reporter amino acid like tryptophan that possesses distinct ultraviolet absorption and strong fluorescence patterns. Finally, it may be possible to modulate the release of a protein in its native form by incorporating certain amino acid sequences between the polymer and protein that are cleaved by specific proteases.

5.4.2.5 PEGs for Reversible Modification

Sometimes PEG modification is the most effective way to enhance the pharmacological properties of proteins, but PEGylation can also decrease a protein's activity to unacceptable levels. In order to maximize all of the benefits associated with protein PEGylation, different approaches were developed. As mentioned in a previous section, the activated PEG double-esters like SS-PEG and SG-PEG contain internal ester bonds that are susceptible to hydrolysis. In addition, benzotriazole and imidazole activated succinic doubleesters were among the first reported PEGylation reagents for reversible amine modification.¹⁷² This approach of reversible PEGylation can be advantageous because the biological activity of a protein is normally restored upon ester hydrolysis, but these polymers leave potentially immunogenic succinyl and glutaminyl groups attached to the protein after their removal. Research has shown these unstable PEG linkers may lead to what is referred to as a hapten, where a group is recognized as an immunogen due to its attachment to a protein carrier. The end result is unwanted immune responses against the hapten group and facilitated clearance of the tagged protein from circulation. In addition, proteins tagged with these groups are inevitably more negatively charged and therefore may be nonspecifically sequestered by the liver and RES to a greater extent than their native or PEGylated counterparts.¹⁷³ More recently, reversible PEGylation of proteins was accomplished by attaching a cleavable oligo-lactic acid PEG to granulocyte colony-stimulating factor (G-CSF).¹⁷⁴ When these conjugates were incubated in pH 7.4 buffer at 37°C for 2 days the biodegradable lactic acid esters hydrolyzed and liberated free G-CSF. However, this reversible PEGylation system likely left some lactic acid residues attached to the protein due to the carbamate linkage between the protein and polymer being hydrolytically stable. It is also likely that attached lactic acid groups are capable of eliciting unwanted immunological responses when they remain attached to proteins.

In order to avoid the potential loss of activity and immunogenic nature associated with the PEGylation of proteins with the reversible double-ester or oligo-lactide PEG reagents mentioned above, different reagents have been developed that are capable of releasing proteins in their native form. Figure 5.9 through Figure 5.12 provide a few examples of these fully reversible PEG reagents, along with their mechanisms of release. PEG-methylmaleic anhydride was the first example of such a reversible PEGylation reagent reported.¹⁷⁵ When this reagent was attached to the amino groups of tissue plasminogen activator and urokinase, both of the conjugates possessed clearance rates that were $5-10 \times$ slower than their native counterparts. In addition, both proteins were released in a completely deacylated form after incubation under physiological conditions (i.e., pH 7.4 and 37°C) for 44 hours. Another category of reversible PEG values of releasing proteins in their native form is based on substituted PEG-phenyl NHS carbonates and these reagents have the advantage of exploiting three distinct mechanisms. When PEGphenylether succinimidyl carbonates or PEG-benzamide succinimidyl carbonates were attached to the amino groups of a model protein, both of the resulting conjugates regenerated the native protein under physiological conditions via a hydrolysis mechanism.¹⁷⁶ Interestingly, the rate of protein release from conjugates prepared using these reagents could be controlled to some extent by the substitution position of the phenyl moiety. Another type of



FIGURE 5.9 Attachment and release of PEG-double esters from proteins (Y is an aliphatic or aromatic moiety). Adapted from Roberts et al., *Adv. Drug Del. Rev.*, 2002; 54: 459.

substituted PEG-phenyl NHS carbonate was developed for reversible modification that releases proteins by a 1,6-elimination mechanism (Figure 5.11).¹⁷⁷ When this type of PEG reagent was used to modify lysozyme via attachment to its amino group(s) the unacylated protein released into PBS or rat plasma was fully ($\sim 100\%$) active. In addition, the rate of protein release was dependent on the degree of modification, temperature, and pH. A final example of a substituted PEG-phenyl NHS carbonate for reversible PEGylation was proposed, which released amino-containing lipids (PE) in their native form via a thiolysis mechanism.¹⁷⁸ The linkage between polymer and amine employed a p- or o-disulfide of a benzyl urethane whose release rate depended on the position of substitution (o > p), but not on solution pH. All of the releasable PEGylation reagents mentioned here might permit the extensive modification of proteins without the corresponding losses in activity frequently seen when permanent modifying reagents are used. However, to date these releasable PEGylation strategies have not produced a candidate for clinical development bringing the relevance of this approach into question.

5.4.2.6 Heterobifunctional PEGs for Tethering, Cross-Linking, and Conjugation

Heterobifunctional PEG reagents are flexible and biocompatible linkers frequently used to covalently join two entities together by reaction with their



FIGURE 5.10 Attachment and release of (A) PEG-methylemaleic anhydride and (B) PEG-phenyl NHS carbonates from amine containing drugs by hydrolysis [X is O or -CONH-]. Adapted from Roberts et al., *Adv. Drug Del. Rev.*, 2002; **54:** 459.

dissimilar terminal groups. These macromolecular spacers can be prepared with numerous classes of complementary terminal functional groups and have been employed in a variety of situations, some of which are listed in Table 5.5. Furthermore, the most popular species of heterobifunctional PEGs have unique combinations of NHS esters, amine, carboxylic acid, vinyl sulfone, maleimide, pyridyl disulfide, silane, vinyl, biotin, or phospholipid terminal groups. The simplest and probably least effective approach for preparing heterobifunctional PEGs is though limiting the proportions of activating reagent (e.g., phosgene, sulfonyl chloride, *p*-nitrophenyl chloroformate, imidazolyl formate, succinic anhydride, etc.) in order to modify a single terminal hydroxyl group of diol-PEG.¹⁷⁹ This approach will almost certainly



FIGURE 5.11 Attachment and release of PEG-phenyl NHS carbonates from amine containing drugs by 1,6-elimination.

produce complex mixtures of non-, mono-, and di-functionalized species from which purification of the favored monofunctional species is extremely difficult. A more sophisticated approach entails initiating the polymerization process with benzyl alcohol, followed by quantitative conversion of the terminal hydroxyl to a specified functional group using chemical methods.¹⁸⁰ Upon subsequent removal of the benzyl group by hydrolysis of hydrogenolysis, the remaining terminal hydroxyl group can be converted to any number of functional groups employing similar methods to those used for conversion of



FIGURE 5.12 Attachment and release of PEG-phenyl NHS carbonates from amine containing drugs by reduction.

the first hydroxyl group. This approach is very efficient but reliant on completely anhydrous polymerization conditions to eliminate diol-PEG impurities, particularly when high molecular weight species are intended. Finally, the ethylene oxide polymerization process can be initiated by an anion that ultimately becomes one of the terminal functional groups accompanied by conversion of the lone hydroxyl group to a functionalized species.^{181,182} This strategy also requires completely anhydrous polymerization conditions to minimize the diol-PEG impurities and is limited to anionic initiators with preferred end groups.

5.5 CONJUGATION AND CROSS-LINKING STRATEGIES

5.5.1 GENERAL CONSIDERATION FOR PROTEINS AND PEPTIDES

Chemical conjugation and/or cross-linking of proteins using PEG is fundamentally dependent on the availability and compatibility of reactive functional groups on both the protein and the modifying reagent. If both classes of functional groups are either not available or compatible, then there is no chance for chemical reactions to occur. Moreover, the outcome of a modification reaction largely hinges on the relative reactivity of a protein's (natural or introduced) constituent functional groups and the specificity of the functionalized PEG In most cases, the biological activity of a protein in a

TABLE 5.5

Categories of heterobifunctional PEGs based on application and nature of their complementary functional groups

Application

Attachment of targeting ligands to natural and synthetic polymers, liposomes, nanoparticles, microparticles, and viruses

Grafting of recognition moieties to surfaces and proteins for biosensors, immunoassays, affinity separations, molecular probes, etc.

Synthesis of enzymatically active cofactor-apoenzyme conjugates Immobilization of enzymes within acrylic polymers and degradable hydrogels Preparation of graft polymeric supports for peptide synthesis

Complementary functional groups

For reaction with carboxylates and amines HCl*H₂N-PEG-CO₂H Boc-HN-PEG-CO₂-NHS Fmoc-HN-PEG-CO₂-NHS

For reaction with sulfhydryls and amines VS-PEG-CO₂-NHS MAL-PEG-CO₂-NHS

For attachment of ligands to materials Biotin-PEG-CO₂-NHS for interaction with avidin/streptavidin containing molecules CH₂=CH-PEG-CO₂-NHS for copolymerization Silane-PEG-NHS for glass substrates Epoxy-PEG-NHS for polymeric substrates Thiol-PEG-NHS for self-assembled monolayers (SAMs) on metal substrates Phospholipid-PEG-NHS for liposomes

conjugated product must be preserved and therefore only those amino acids not essential to its biological functions should be targeted. Importantly, the threedimensional conformation of a protein should not be significantly perturbed after polymer conjugation because any disturbances in tertiary structure caused by changes in size, charge, or other characteristics will likely diminish the conjugate's ability to function properly.

Provided here is an account of the factors that should be considered before any attempts are made at modifying a protein with PEG, along with a general list of the most commonly utilized protein PEGylation strategies. A protein's solubility; thermal, pH, mechanical, and proteolytic stability; molecular size and charge; immunogenicity and antigenicity; and biological activity and pharmacokinetics are all affected by PEG modification. Thus, identifying the desired outcome(s) of PEGylation can simplify the conception of rational and effective modification strategies, based on determining which of these properties are the most important to modulate and which are the most

TABLE 5.6 Modification strategies used to maximize the activity of PEG-protein conjugates

Nonspecific amine modification

Attaching a minimum number of PEG chains via stoichiometric deficiency Controlling reaction environment for directed modification based on differing pK_a values of available amino groups Using less reactive PEG reagents, lower temperatures and longer reaction times to manipulate reactivity versus selectivity under certain reaction conditions Attaching a few branched or high molecular weight PEG chains **Selective modification** Site-directed N-terminal conjugation with PEG-aldehyde Conjugation to the carbohydrate groups of glycoproteins Sulfhydryl-selective conjugation Site-directed (lysine/cysteine) mutagenesis Site-specific protection/deprotection On-column derivatization Peptide synthesis using PEG supports Enzyme-mediated coupling

(Adapted from Gombotz and Petit, American Chemical Society, 2000.)

important to conserve. Careful examination of a protein's primary sequence, and understanding of each functional group's relative reactivity and involvement in biological function(s) can greatly aid in the design of modification procedures that yield conjugates with advantageous properties. This is necessary because the same mechanism (i.e., steric shielding) that protects proteins from recognition and elimination by proteolytic enzymes and antibodies can also prevent the adverse effect on the PEG-modified protein's biological function(s).

Knowledge of a protein's functional classification (e.g., enzyme, cytokine, antibody, hormone, etc.) will help in determining what approaches are most likely to produce the desired outcome(s). For example, protein hormones and cytokines are often ligands for specific cellular receptors and increasing the extent of PEG modification or polymer molecular weight may diminish the conjugate's biological activity by interfering with the requisite receptor–ligand interactions. Oppositely, enzymes, receptors and antibodies are often proteins involved in ligand recognition, and it is conceivable that the attachment of larger PEGs or greater degrees of modification can be tolerated without substantially reducing the conjugates' functional capabilities. PEG modification strategies are generally classified as either nonspecific or selective. Table 5.6 lists some general approaches that can be used to maximize the biological activity of PEG-protein conjugates.

For proteins that have (on average) one PEG chain attached to it via a nonspecific method, the polymer can be present at a number of different sites, resulting in complex mixtures of positional isomers. The number of positional isomers (P) differing in their site(s) of PEG modification can be estimated according to the following relationship:

$$P = \frac{N!}{(N-k)! \times k!}$$

where N is the number of possible modification sites (amino groups) and k is the number of sites modified. Thus, for lightly modified species, P increases with the extent of nonspecific protein modification. The importance in minimizing P lies in the fact that, oftentimes, each positional isomer will contribute its own distinct physicochemical, biological, and pharmacological properties to the overall properties of the heterogeneous PEG-protein mixture.

5.5.2 NONSPECIFIC MODIFICATION

The most prevalent approach employed to modify proteins with PEG is the reaction of the protein's alpha- and/or epsilon-amino groups with electrophilically activated PEG derivatives. This method has flourished because proteins often contain numerous lysine residues (sometimes exceeding 10% of the total sequence) and these residues typically reside in (or near) the solventaccessible regions also available to modifying reagents. When the amino groups of proteins are PEGylated in a nonspecific manner it is not uncommon for several (or all) of these groups to be modified. Such nonspecific PEGvlation schemes create heterogeneous mixtures of PEG-protein species with varying degrees and sites of modification. Depending on the location of the modified amino groups relative to the protein's functional surfaces, the nonspecific attachment of PEG may mask certain areas important for activity without masking those areas recognized by antibodies or proteolytic enzymes, creating a conjugate with sub-optimal characteristics. For example, PEGylated derivatives of epidermal growth factor (EGF) were prepared by reacting the protein with a 30× excess of SC-PEG in pH 8.0 PBS.¹⁸³ The mono-PEGylated species were then isolated from a complex mixture of reaction species by sizeexclusion chromatography. Reversed-phase chromatography was then used to separate the different positional isomers for further characterization. Indeed, it was found that the activity of the mono-PEGylated EGF conjugates was highly dependent upon the site of substitution, with the N-terminally modified species possessing the greatest activity and subsequent work was focused on preparing this species in high yield. The approach outlined above can be considered a practical way of preparing conjugates for further characterization, and once the preferred species have been identified, different methods of conjugation may be employed to maximize reaction yields of conjugates with the most advantageous properties.

Ideally, the reactive amino acid side chains to be used as "handles" in a conjugation reaction would be found on the protein's surface and consequently accessible to the appropriate functionalized PEG allowing for efficient modification under relatively mild conditions. For example, if charged side chains are the targets for PEG modification, then aqueous reaction systems may be used with the assumption that the targeted residues will lie at (or near) the protein-water interface. In addition, the average number of PEG chains attached to a protein can be restricted in a general sense by adding stoichiometrically deficient amounts of PEG reagent relative to the number of reactive protein amino groups. PEGylated human growth hormone (hGH) was modified in such a way, where SCM-PEG was reacted at a 1:1 ratio (to the 10 available primary amines) in pH 8.5 borate buffer.⁷⁶ Following an initial polishing step and subsequent fractionation of the individual PEGmers by cation exchange chromatography, the reaction product was found to contain 4-5, 5, 5-6, or 6 PEGs/protein. Fortunately, each of these species was active and no further optimization of the reaction conditions was deemed necessary for the preparation of conjugate with favorable properties.

If inaccessible (i.e., sterically crowded) residues are the targets for PEG conjugation, then organic reaction systems can be selected in which these normally buried residues are exposed to the protein-solvent interface making them more convenient sites for modification. Here it should be noted that any selectivity afforded by the minute differences in microenvironment between (otherwise equivalent) lysine side chains is normally lost when the protein's structure is perturbed upon addition of some organic solvents (e.g., DMSO, DMF, NMP, etc.). When insulin was randomly PEGylated in DMSO, the resulting product maintained full biological activity following intravenous or intratracheal administration.¹⁸⁴ On the other hand, certain organic solvents can actually stabilize a protein's structure and, consequently, the microenvironments from which the unique pK_a values of equivalent lysine side chains originate are also stabilized. It is not uncommon to see aqueous/organic cosolvent (e.g., buffer-ethanol, DMSO-water, DMF-water, etc.) systems utilized in order to access amino acids that lie somewhere between a protein's hydrophobic core and its surface. For example, insulin was modified at one of its normally unreactive (due to steric crowding in aqueous buffer) primary amino groups. In a basic aqueous/organic solvent system (60% DMF, $pH_{app} \approx 9.0$), the amino group of interest (N^{eB29}) was modified at a high conversion (>80%) without affecting the proteins' activity.⁸³ Thus, the appropriate choice of solvent systems can be used in conjunction with pH control (see Section 5.3.2) to aid in directing the modification reaction towards those amino acid(s) least likely to disrupt the biological functions of PEGprotein conjugates.

The size and/or shape of the PEG reagent can also greatly affect the selectivity of conjugation reactions based on steric constraints. For example, very large or branched PEG reagents cannot easily access lysine side chains that are in sterically crowded environments. Therefore, if the target amino acid is in a hindered position it may be necessary to use linear and low molecular

weight modifying reagents to access these residues. An example of this sort of strategy is illustrated in the case of a PEGylated interferon-beta (IFN- β) derivative.⁹⁸ Here the low molecular weight homobifunctional reagent PEG-OPSS₂ ($M_r = 2000 \text{ Da}$) was used to selectively modify a cysteine sulfhydryl group found to lie proximal to the protein surface, but sterically "hidden" from larger modifying reagents. A larger PEG-thiol reagent ($M_r = 2000 \text{ Da}$) was then attached to the low molecular weight PEG spacer resulting in a conjugate with preferred properties. Conversely, some amount of specificity can be conferred to modification schemes if the target amino acids are unhindered relative to so-called "interfering" amino acids and large/bulky PEG reagents are used. In the case of a PEGylated inferferon-alpha (IFN- α -2a) derivative, reaction with a low molecular weight ($M_r = 5000 \text{ Da}$) activated PEG resulted in 11–15 positional isomers, whereas conjugation with a large branched PEG species ($M_r = 40,000 \text{ Da}$) limited the number of positional isomers to four.^{95,185}

5.5.3 SELECTIVE MODIFICATION

Selective approaches to protein modification have long been recognized as a means of producing active conjugates possessing favorable characteristics. In theory, certain reaction conditions should favor the modification of particular lysine groups by generally adhering to the reactivity/selectivity principle. Such a manipulation of conditions is usually in the form of applying less reactive PEG derivatives, in conjunction with low reaction temperatures and long reaction times to direct the chemical reactions to only those lysine amino groups most favored to react based on their individual pK_a values. This approach lies at the heart of selectively modifying proteins at their N-terminal amino group(s) with aldehyde-PEG derivatives. For example, a PEGylated version of granulocyte-macrophage colony-stimulating factor (GM-CSF) was prepared via reductive amination of the cytokine's N-terminal amino group using PEG-propionaldehyde $(M_r = 6 \text{ or } 42 \text{ kDa})$.⁶⁹ The conjugation reaction was carried out at 0 to 4°C for 2 weeks and it was determined that the N-terminally mono-PEGylated GM-CSF conjugates had increased in vivo activities relative to native protein. In addition, a PEGylated version of a glycosylated form of IFN- β (IFN- β -1b) was prepared using a 20 kDa branched PEG-Ald and pH 6.0 to selectively modify the cytokine's N-terminal amino group.⁹⁹ The resulting conjugate's site of substitution was confirmed by peptide mapping and its antiviral activity was found to be identical to the native species. Finally, PEGylated versions of granulocyte colony-stimulating factor (G-CSF) and consensus interferon (C-IFN) were prepared using aldehyde-PEG derivatives. These conjugation reactions were carried out under slightly acidic conditions (pH 5) at 4°C.⁶⁶ In both cases the conjugate reaction mixtures contained >80% of the intended (N-terminally alkylated) positional isomers. In fact, numerous reports of N-terminally alkylated PEG-protein conjugates have been published in the last decade and the PEG-G-CSF conjugate currently approved by the FDA for human use was prepared using this selective approach.

An alternative approach to selective N-terminal modification of proteins with PEG aldehyde derivatives involves the attachment of nucleophilic PEG derivatives to aldehyde groups of oxidized glycoproteins. Since the regions of glycosylation contained in glycoproteins are usually well removed from the protein's active site and these positions are readily accessible, they represent excellent targets for the selective attachment of PEG.¹⁸⁶ For example, because antibodies are predominantly glycosylated in their *Fc* regions these oligosaccharide groups can be oxidized by periodate treatment and selectively modified with nucleophilic reagents such as PEG-hydrazide. Furthermore, N-linked glycosylation sites have been deliberately introduced into proteins with the purpose of providing polysaccharide targets for PEGylation.¹⁸⁷

A more common approach employed to selectively modify proteins with PEG is to target cysteine sulfhydryl groups using thiol-specific PEG reagents. The selectivity of this approach depends on the scarcity of cysteine residues on the surfaces of proteins, due to their relative hydrophobicity. In addition, genetic engineering can insert cysteine residues at specific areas in the protein sequence not expected to interfere with the protein's function. In this way, cysteine selective PEG reagents can be used to site-specifically modify proteins at areas on the protein surface that confer the beneficial properties commonly associated with PEGylation without unnecessarily minimizing the conjugate's activity. For example, studies with PEGylated (monovalent) Fab' antibody fragments have shown that conjugates prepared in this manner retained full antigen binding capabilities, even when the molecular weight of the modifying polymer reached 40 kDa.¹⁸⁸ In addition, when the native glycosylation site of recombinant human interleukin-2 (rhIL-2) was substituted with a cysteine residue followed by selective PEGylation, the conjugate retained full activity.¹⁶⁷ Furthermore, when a PEGylated version of the highly potent (although highly immunogenic) anti-HIV protein cyanovirin-N was sought, nonspecific modification resulted in a total loss of antiviral activity. However, when a cysteine residue introduced by site-directed mutagenesis and subsequently modified using a cysteine selective PEG reagent, the conjugate remained highly active.¹¹⁷ Finally, the selective modification of protein amino groups with PEG can be achieved by applying genetic engineering techniques. Site-directed mutagenesis has been used to substitute undesirable lysine residues with arginines or to introduce lysine groups in favorable positions.189,190

A relatively sophisticated strategy that has been employed to modify selected amino groups in proteins with PEG is based on the reversible protection of interfering nucleophilic groups using methods common in protein chemistry. Chemically protected intermediates of insulin, vapreotide, and tumor necrosis factor-alpha (TNF- α) were all selectively PEGylated with favorable results over the corresponding conjugate species prepared using nonspecific methods. A large body of literature exists establishing the N-terminal amino group of insulin's B-chain (N^{α B1}) as the most favorable site for PEG modification.^{77,79,82,191} Insulin conjugates modified at residue PheB1 are highly stable, and nonimmunogenic, and fully active species with prolonged residence times in circulation.⁸⁴ However, the PheB1 amino group is typically the least reactive towards electrophilically activated PEG reagents under the basic conditions requires for coupling due to steric constraints. Thus, the two competing nucleophilic amino groups (i.e., GlyA1 and LysB29) were acylated with a removable protecting group prior to PEG attachment, enabling the site-specific conjugation of insulin at the preferred PheB1 amino group in high yield. Subsequent removal of the protecting groups afforded the fully active insulin conjugate with the most advantageous properties.

Vapreotide (an eight-amino acid somatostatin analog) has also been sitespecifically PEGylated using a protection-deprotection scheme.⁸⁶ In this work, either the N-terminal α -amino or lysine ε -amino group of vapreotide was reversibly protected prior to reaction with an alkylating or an acylating PEG reagent. Four different mono-PEGylated vapreotide species (i.e., N-terminally alkylated, N-terminally acylated, lysine-alkylated, or lysine-acylated) were prepared and characterized, and their individual biological activities were assessed. This research showed the modification of the N-terminal amino group substantially altered the peptide's conformation, while the preservation of the lysine group's positive charge via reaction with an alkylating PEG derivative (i.e., Ald-PEG) was necessary for in vitro activity. Thus, site-specific PEGylation of this peptide was required to obtain useful information on the identity of the most preferred species, enabling further efforts to be directed towards preparing this specific conjugate species in high yield.

Highly active PEG modified TNF- α derivatives have also been prepared utilizing a reversible-protection scheme.¹⁰⁰ These conjugates provide an excellent example of how this selective modification approach can be applied to larger and more complex proteins. Of course, in this case the degree of selectivity is naturally lower due to the character of the TNF- α cytokine, which contains 18 lysine ε -amino groups available for modification with PEG Therefore, it would be extremely difficult to specifically protect the only those amino groups involved in the cytokine's function without also inadvertently blocking some of the targeted sites (i.e., lysine amino groups) for PEG modification. In this case the protected TNF- α intermediate was prepared by reaction with a pH-reversible amino-protective reagent, dimethylmaleic anhydride (DMAAn), resulting in the protection of about 35% of the available lysine amino groups. PEGylation of the remaining free amino groups and subsequent deprotection of the PEGylated construct afforded the final product. In vitro studies indicated that the conjugates prepared via the protected intermediate possessed 20-40% higher specific activities than the conjugates prepared using a nonspecific PEGylation approach. In addition, the in *vivo* potency of the selectively modified PEG-TNF- α conjugate was found to be $30 \times$ higher and $2 \times$ higher than that of the unmodified and nonspecifically modified cytokines, respectively.

An alternative method to the selective modification of larger proteins with PEG involves the use of "on-column" conjugation methods. In this approach, proteins immobilized on liquid chromatography supports (e.g., ion-exchange,
affinity, etc.) are modified with activated PEG reagents to increase the selectively of PEGylation. Etanercept, a chimeric protein where the extracellular domain of p75 tumor necrosis factor receptor (TNFR) is linked to the Fc portion of a human IgG1 antibody, was recently approved for human use in the treatment of rheumatoid arthritis. Conjugation of SC-PEG ($M_r = 5000 \text{ Da}$) to etanercept was investigated as a way of increasing its circulation half-life, and ultimately decrease the dosing frequency and total dosing required for therapeutic efficacy.¹⁹² Prior to PEG modification, etanercept was loaded onto an affinity column presenting an immobilized TNFR: Fc neutralizing antibody. When etanercept was nonspecifically PEGylated in solution without the aid of the affinity column, the number of PEG chains per protein molecule and conjugate molecular weight increased. Unfortunately, increases in the extent of conjugation and molecular weight were inversely related to the biological activity of conjugate. In fact the in vitro activity of the "on-column" modified PEG-etanercept was always >100% relative to the native protein. Apparently, by interacting with the amino acid side chains important to the protein's activity, the neutralizing antibody prevented these selected residues from reacting with the PEG reagent.

Chemically synthesized peptides can be site-specifically modified with PEG in a number of ways.⁸⁹ PEG supports can be used in the preparation of the peptides, with the desired peptide easily isolated in its PEGylated form. Conditions have been developed for N-terminal, side chain, and C-terminal modification of synthetic peptides prepared using a solid-phase approach. In addition, solution phase conditions have been developed for the site-specific PEGylation of peptides utilizing an introduced cysteine residue that is subsequently modified with any of the appropriate reagents mentioned previously.

Finally, enzyme-mediated coupling is another method for the site-specific modification of proteins with PEG.¹⁹³ Here, a genetically engineered glutamine aminotransferase is used to selectively attach PEG to glutamine residues contained within a recognized substrate sequence. Such substrate sequences, derived from known substrates found in Substance P and fibronectin, can be genetically introduced into regions of a protein known not to interfere with its function. Site-specifically PEGylated IL-2 conjugates were prepared in this way had well preserved *in vitro* activities (relative to randomly PEGylated conjugates) and increased half-lives relative to the native protein.

5.6 PURIFICATION AND CHARACTERIZATION OF PEG-MODIFIED MACROMOLECULES

5.6.1 PURIFICATION

A variety of techniques have been used to purify PEGylated proteins from their unmodified counterparts. These techniques exploit changes in the native protein's size, charge, hydrophilicity, hydrophobicity, or solubility resulting

from PEG attachment. Conceptually, there are two degrees of conjugate purification: (1) isolation of the PEGylated species from low molecular weight reagents (leaving groups, organic solvents, buffer salts, acids, bases, etc.), unreacted polymer and unmodified protein; and (2) separation of the individual PEGylated species (i.e., PEGmers and/or positional isomers) from each other. The former is relatively uncomplicated and easy to achieve, while the latter can be extremely difficult (if not impossible) to accomplish. When sufficient differences in molecular weight exist, conjugates can be purified using dialysis or ultrafiltration. Examples of such a fortunate circumstance were seen in the case of PEGylated versions of epidermal growth factor (EGF) and interferon-alpha (IFN- α) that were purified using only dialysis.^{85,194} However, if the differences in molecular weight between conjugate, polymer and protein species do not permit efficient purification using the simple approach mentioned above, then more discriminating size-based techniques can be applied. Size exclusion chromatography (SEC) is frequently employed to efficiently purify conjugate species from starting materials (i.e., free PEG and protein) based on their molecular sizes. As mentioned in a previous section, it is likely that each of the PEG-protein species (i.e., PEGmer's and/or positional isomers) contained in a heterogeneous reaction mixture will have distinct physicochemical, pharmacological, and immunological properties. Therefore, it may be necessary to isolate the species with favorable characteristics from those with unfavorable ones. Preparative SEC can be adapted to separate and purify conjugates that differ from one another in the number of attached PEG chains, but this technique suffers from an extremely low loading capacity (on the order of 1 mg protein/60 mL resin). In addition, SEC is not a practical way of isolating positional isomers with the same degree of modification, and therefore differences in other molecular properties must be exploited.

The nature of the chemistry used to modify proteins with PEG frequently determines which techniques are appropriate for conjugate separation and purification. For example, when PEG modification changes a protein's overall charge and/or charge density (e.g., amino group acylation or carboxyl group amidation), ion-exchange chromatography can effectively be utilized even when there are no net charge differences between components. When ionexchange chromatography is used to separate and purify PEG modified proteins a conjugate's retention time generally is inversely proportional to its degree of modification. Pure fractions of both mono- and di-PEGylated GM-CSF conjugates have been separated from a reaction mixture containing the conjugates, free PEG and native protein using anion exchange chromatography based on the ability of high molecular weight PEG attachment to shield charges on the protein surface.⁶⁹ In addition, eleven distinct positional isomers of a mono-PEGylated interferon α -2a derivative have been separated and purified by cation exchange chromatography using an ascending pH (4.3–6.4) gradient.95 Furthermore, each of the three possible positional isomers of mono-PEGylated insulin have been isolated in a highly pure state from a heterogeneous mixture also containing di- and tri-PEGylated species by cation

exchange chromatography using an increasing salt (0.01-0.3 M NaCl) gradient.¹⁹⁵ A significant benefit of using, i.e., X to isolate and purify PEGprotein conjugates is the large loading capacities of the resins (up to 80 mg protein/mL resin) relative to those used in other techniques.¹⁹⁶

If size- or charge-based techniques are not capable of adequately separating and purifying PEG-proteins, as is often the case when thiol-selective or alkylating PEG reagents are used, then minute differences in hydrophobicity between the conjugates and starting materials can be taken advantage of using RP and HIC.^{102,128} RP-HPLC and HIC separation of PEG-protein conjugates both depend on the hydrophobicity of the protein relative to PEG.¹⁹⁷ Reversephase techniques for protein purification and analysis are commonly encountered in the biochemical sciences, and therefore will not be expanded upon in this chapter. However, because HIC is less-frequently covered, an examination of its advantages and disadvantages is provided here. Like RP, the stationary phases in HIC are hydrophobic, although considerably more hydrophilic than the octadecyl (C_{18}) hydrocarbon bonded phases commonly used in RP. HIC's mechanism (or mode) of separation stems from the hydrophobic effect, whereby hydrophobic materials are retained when the salt concentration is high and elution is achieved by decreasing ionic strength.¹⁹⁸ HIC relies on the ability of concentrated salt solutions to stabilize a protein's tertiary structure, allowing for its purification with retention of activity and HIC is highly sensitive to the effects of protein modification with Pe.g. Overall, HIC is very well suited to purifying PEGylated proteins that are unstable in the aqueous-organic mobile phase systems required for RP-HPLC, while RP-HPLC is particularly effective in purifying PEGylated peptides because they are oftentimes soluble and stable in such systems. Numerous PEGylated versions of vapreotide (a somatostatin analog) and growth hormone-releasing factor (GRF) analogs were purified using RP-HPLC with good recoveries of conjugates whose activity was not negatively affected by the purification process.^{86,88} Interestingly, PEG-peptides ordinarily elute from RP columns after the free peptide where the increased retention is often directly proportional to the conjugates' degree of modification and the same order of elution is seen using HIC.^{89,199} A limitation to using HIC for purification of PEGylated proteins is the use of high salt concentration solutions, which cannot support high concentrations of hydrophobic materials. Therefore the sample volumes and concentrations used are often prohibitive to process scale-up.

Affinity chromatographic methods have also been utilized to purify PEG modified protein conjugates. These columns differentiate between conjugate species based on their unique affinities for prosthetic groups immobilized to resin beads. For example, PEGylated antibodies and receptors can be purified using affinity columns presenting the appropriate antigens and ligands, respectively. However, a major shortcoming of the specific affinity-based separations lies in the recurrent lack of adequate amounts of material available for stationary phase immobilization that result in (sometimes insurmountable) obstacles to process scale-up.

Finally, it is often necessary to use a combination of the previously mentioned techniques to adequately separate and purify specific PEGylated protein conjugates. SEC and X chromatographic techniques were used in sequence to remove native protein and unreacted polymer from a PEGylated IFN- β preparation, respectively.⁹⁹

5.6.2 CHARACTERIZATION

5.6.2.1 Degree of Substitution

Characterizing the products of a PEG modification reaction is difficult because the (frequently heterogeneous) mixture contains a family of species distinguished both in their number and positions of PEG attachment and the situation is further complicated by the inherent polydispersity of the polymer. For example, when a protein such as myoglobin (contains 20 reactive amino groups) is modified with a 10 kDa PEG having a polydispersity of 1.2 can result in a mixture of up to 10³³ different molecules.²⁰⁰ Another confounding factor is the fact that PEG itself is optically transparent and nonfluorescent, and therefore undetectable using conventional spectroscopic methods.²⁰¹ One approach to determining the degree of modification involves estimating the amount of PEG contained in a conjugate by colorimetric reaction with iodine, but this method is very insensitive because of the high signal provided by the blank.²⁰² More recently, another colorimetric method with far greater sensitivity (down to 5µg PEG) was developed for quantitatively measuring the quantity of PEG in PEG-protein conjugates. This assay measures the amount of the chromophore [Fe(SCN)₃] that partitions from aqueous ammonium ferrothiocyanate solutions into methylene chloride with the presence of PEG in a concentration dependent manner.²⁰³ However, this method's reliability in determining a conjugate's degree of substitution fundamentally depends on complete removal of free PEG prior to assay, otherwise artificially high values owing to free PEG are unavoidable.

As mentioned previously, the most commonly utilized approach to protein PEGylation involves the modification of amino groups (α and ε) with either alkylating or acylating PEG reagents. The degree of substitution in this class of conjugates is most often characterized using methods that determine the number of free lysine groups relative to a parental protein standard, with increasing degrees of modification indirectly measured by decreasing numbers of free amino groups. The Habeeb method is a classical procedure where unmodified lysine amino groups are reacted with trinitrobenzenesulfonic acid (TNBS) followed by UV quantification.²⁰⁴ Unfortunately, the Habeeb method is rather insensitive and nonspecific in nature, only being able to report the average number of free amino groups per milligram of protein. Additional liabilities inherent to this method include the necessity for protein concentration determination using other techniques (e.g., the biuret method) and the negative influence that free-PEG can have on assay results. A superior approach to determining the average number of modified amino groups in

a conjugate is the fluorescamine method.¹⁶³ This method works by reaction of fluorescamine with the unmodified protein amino groups resulting in a fluorescent derivative whose concentration can be directly determined without prior knowledge of protein concentration. The fluorescamine method also has the advantages of greatly increased sensitivity (i.e., nanogram amounts of protein can be used) and no interference caused by free PEG Nevertheless, both the TNBS and fluorescamine methods have proven to be quite useful for monitoring protein PEGylation using lysine-active PEG reagents. Related spectrophotometric methods have been used to determine unmodified cysteine groups based on their reaction with either 2,2'-dipyridyl disulfide (forms 2-thiopyridine with $\varepsilon_{343 \text{ nm}} = 7060 \text{ cm}^{-1} \text{ M}^{-1}$ at pH 7.2) or Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid), $\varepsilon_{412 \text{ nm}} = 14,150 \text{ cm}^{-1} \text{ M}^{-1}$ for thionitrobenzoate anion].^{205,206}

Two more rigorous techniques were developed for determining the extent of protein modification with PEG. In the first method, the type of chemistry used to modify proteins must result in the formation of hydrolytically stable-linkages (e.g., secondary amine, thioether, sulfone, imidyl, etc.) between the polymer and protein. In general, the amino-specific reductive amination and thiol-specific Michael-addition PEGylation chemistries will give rise to conjugates with linkages that survive the extreme hydrolysis (6 N HCl. 100°C, 72h) typically used in Amino Acid Analysis (AAA). The degree of modification of PEG-protein conjugates can then be determined based on the decreased abundance of lysine and/or cysteine groups in the hydrosylate. The second AAA-based method for determining an overall extent of modification for a conjugation product requires the use of specific PEG reagents containing an unnatural amino acid residue or peptide sequence (e.g., norleucine or β -alanine) as a spacer arm between the polymer and protein. 35,207Here, the polymer is cleaved from the protein during acid hydrolysis leaving the reporter group behind for quantification. Furthermore, the branched PEGs built from a lysine core can also be characterized in this way, but it may be difficult to identify low levels of modification solely using this method.

Other methods for determining a conjugate's average degree of modification include proton nuclear magnetic resonance (¹H-NMR) and phase partitioning of the conjugate in aqueous two-phase systems.^{128,208} The former takes advantage of the single, sharp signal at $\delta = 3.7$ ppm for the direct determination of mPEG content and the concentration of mPEG in the conjugate can be calculated within 2% by comparison with appropriate standards. The latter takes advantage of the relation between a conjugate's degree of modification and its phase partitioning in aqueous two-phase systems of PEG and dextran, where the attachment of PEG chains to a protein increases its partitioning into the PEG-rich top phase. Upon measurement of the protein concentration on both phases, a linear relationship between log partition coefficient and the number of PEG chains bound is used to sensitively measure PEG-modification. However, both techniques are nonspecific yielding average degrees of PEG-modification unless the conjugate preparation has been purified beforehand.

Many of the chromatographic techniques used to separate and purify PEG modified proteins can also be utilized for the purpose of conjugate characterization. Analytical HPLC can also be used to examine PEG-protein conjugates based on size, charge, or hydrophobicity differences between PEGmers. HPSEC, HPIEC, RP-HPLC, and HPHIC were all used to characterize the heterogeneity of PEG-modified superoxide dismutase (SOD) preparation.¹⁹⁹ Unfortunately these techniques gave only qualitative information about the conjugate in terms of identifying the different populations of molecules differing in their degree of modification, and the correlation of these data with those obtained using orthogonal methods was necessary for a more far-reaching analysis. For example, HPSEC analysis was able to resolve four different peaks containing PEG-SOD based on size. However, the actual molecular size of the species contained in each peak could not be accurately determined by comparison to conventional protein calibration curves because of the hybrid nature (i.e., globular protein core with a shell of random coil polymers) of the conjugate. In addition, the exact identity (extent of modification) of the conjugate species contained in each peak could not be determined based on their relative elution times from the SEC column, but rather had to be established indirectly by comparison to data generated using a fluorescamine-based method. In this same study RP-HPLC and HPHIC were both able to resolve between the different PEGmers contained in the PEG-SOD preparation based on their different capacities for column retention, but again there was no way to independently confirm the degree of modification of the conjugates contained in each peak. HPIEC analysis did suggest that there was a population of species contained in each of the peaks resolved using the other chromatographic techniques, most likely due to the minute differences in charge density that can exist between positional isomers.

Capillary electrophoresis (CE) is a powerful analytical method that separates molecules on the basis of differential migration of each species in an electric field through a narrow-bore, fused-silica capillary column. Separation of components in a complex mixture can be a function of molecular size, charge, and/or shape. Protein adsorption to the wall of a fused silica capillary frequently occurs due to various forces such as hydrophobic interactions, ionic interactions, hydrogen bonding, and van der Waals forces.²⁰⁹ The nonspecific adsorption of basic proteins and peptide to a capillary's surface silanol groups often limits the efficiency of separation due to peak tailing and/or irreproducible migration times. Numerous methods have been developed to minimize protein adsorption by treating the capillary surface polymers such as Pe.g., polyacrylamide, or cellulose.^{210–213} A free-solution CE method was developed to separate PEG modified protein species according to numbers of attached polymer chains, where greater degrees of modification resulted in longer migration times.²¹⁴ This method employed specific conditions to minimize electroosmotic flow, conjugate adsorption to the capillary wall and any differences in total charge between individual classes of PEGmers, creating a separation mechanism dominated by differences in molecular size. In another study, a charge-reversed CE method was developed in which the capillary

column was coated with a positively charged substance in order to reverse the direction of electroosmotic flow and prevent the adsorption of basic and PEGylated proteins to the capillary wall. The electrode orientation was then reversed resulting in an inverse relationship between the number of PEG chains attached and the conjugate's positive charge and migration time.²⁰⁰ Interestingly, the mechanism of separation for this method was governed both by molecular size and net positive charge of the conjugate. Similar to other techniques used to determine the degree of conjugate modification, the breadth of peak's encountered using either CE method was a reflection of PEG's polydispersity and the heterogeneity of species contained within each class of PEGmers. The possibility does exist for the peak broadening effects of PEG polydispersity to obscure the differences in size between different classes of PEGmers, resulting in the loss of resolution between species. Furthermore, the apparent necessity for separation conditions to be tailored to each protein conjugate of interest may prevent CE from becoming a widely used tool in conjugate analysis.

5.6.2.2 Site of Substitution

Many times a protein will have more than one site available for PEG attachment and this may result in the production of a mixture of PEG-Xprotein (where X is the site of modification) conjugates, even in the case of mono-PEGylated adducts. Sometimes it is possible to separate the individual positional isomers contained in a conjugate mixture, while oftentimes only the different PEGmers (PEG_u-protein) can be separated from each other. In either case, it is usually important to identify the different positions of PEG attachment and amounts of each positional isomer in a conjugate preparation in order to better appreciate the effects of PEGylation on protein' properties. To this end, traditional protein chemistry techniques have been adapted to identify the position of polymer attachment for PEG modified proteins. One popular and extremely sensitive method applies N-terminal protein sequence analysis (Edman degradation or Edman sequencing) to determine the site(s) of PEG attachment. Generally speaking, this mode of analysis can indirectly identify N-terminally acylated or alkylated proteins by their inability to undergo the intramolecular cyclization reaction necessary for sequence analysis or PEG-modified side chains based on the absence of their phenylthiohydantoin (PTH) derivatives in the sequence analysis. Edman degradation involves the reaction of protein α -amino group(s) with phenylisothiocyanate in dilute base, resulting in a thiourea intermediate that activates the adjacent N-terminal peptide bond. Upon mild acid treatment, the amino group of the thiourea attacks the activated peptide bond via a facile intramolecular cyclization reaction that releases the N-terminal amino acid in the form of a PTH derivative. The PTH-amino acid derivative can then be identified and quantified by chromatography (i.e., RP-HPLC), according to retention time and abundance, by comparison with standard PTH derivatives of known amino acids. Following cleavage of the peptide bond, a new N-terminal amino

TABLE 5.7 Enzymes and chemicals used as selective cleavage agents for peptide mapping

Enzyme	Cleavage site(s)
Trypsin	Carboxy side of Arg and Lys
Chymotrypsin	Carboxy side of Phe, Tyr and Trp
V-8 (Staphylococcus aureus protease, Glu-C)	Carboxy side of Glu and Asp
Lys-C	Carboxy side of Lys
Arg-C	Carboxy side of Arg
Asp-N	Amino side of Asp, cysteic acid
Clostripain	Carboxy side of Arg
Pro-C	Carboxy side of Pro
Chemicals	
Cyanogen bromide	Carboxy side of Met
BNPS-skatole [2-(2-nitrophenylsulfenyl)-3-methylindole]	Carboxy side of Trp
Iodosobenzoic acid	Carboxy side of Trp
Formic acid	Peptide bond between Asp and Pro
Hydroxylamine	Peptide bond between Asn and Gly
NTCB (2-nitro-5-thiocyanobenzoic acid)	Amino side of Cys in presence of N

acid is generated that may be identified by repetition of the sequencing procedure and Edman degradation has been used successfully to determine the sequence of proteins' having as many as sixty amino acids. N-terminal protein sequencing is a sensitive technique (i.e., fmol levels of detection) suitable for characterizing the positions of PEG attachment to peptides, and has been used to characterize the positional isomers of PEG modified forms of insulin⁸³ and GRF.⁸⁹

While Edman degradation can sequence polypeptides up to sixty amino acids long, it is often desirable to simplify the analysis of more complex proteins by cleaving the biopolymer into shorter chains and separating the resultant peptide chains using chromatographic (e.g., ion exchange, reverse phase, etc.) techniques. Table 5.7 lists several enzymes and chemical methods commonly used to cleave polypeptide (protein) chains into shorter peptide segments. The former approach is remarkably adaptable because numerous enzymes with unique specificities can be used alone or simultaneously to specifically cleave polypeptides at distinct locations, enabling peptide fragments to be pieced together in a meaningful way for determination of the site(s) of polymer attachment. Two exemplary cases, PEG-IFN- α -2a (Pegasys)⁹⁴ and PEG-IFN- α -2b (PEG-Intron),²¹⁵ have been reported in which the numerous possible positional isomers of mono-PEGylated species contained in a conjugate preparation were characterized by enzyme cleavage in tandem with Edman sequencing. In both cases, the positional isomers were separated using precisely adapted ion-exchange chromatography and the distinct species were subjected to enzymatic cleavage. The individual positional isomer digests were then fractionated by chromatography (RP or SEC) and analyzed by N-terminal sequencing. This multi-step approach to conjugate characterization permitted the accurate identification of each positional isomer contained in the complex mixture of mono-PEGylated species. Furthermore, identification of the single positional isomer of a selective PEG-anti-IL-8 antibody conjugate was confirmed using the peptide cleavage, fragment separation, N-terminal sequencing multi-step approach.¹¹⁴ However, it should be noted that the hindrance of the bound polymer chains could prevent the specific cleavage by enzymes and incomplete proteolysis near the site(s) of PEG attachment is expected. In addition, problems related to isolating and purifying the individual products of the digestion might occur.

As mentioned above, several chemical methods common to protein chemistry can also be used to cleave polypeptide (protein) chains into shorter peptide segments. In general, such chemical methods are not affected by the presence of bound polymer chains and are therefore very useful alternatives to proteolytic digestion of conjugates. However, the same chemical reactions that cause polypeptide fragmentation may also destroy the polymer and the success of this approach is necessarily determined on a case-by-case basis. The most frequently adopted procedure utilizes cyanogen bromide to specifically cleave polypeptides at methionine residues. This method commonly produces a manageable number of peptide fragments for N-terminal sequencing because proteins contain relatively few methionine residues. Alternatively, activated polymers with peptide spacer groups containing methionine and an unnatural amino acid (e.g., norleucine or β -alanine) can be used to facilitate the identification of the site(s) of polymer attachment in protein conjugate. Following removal of the polymer by treatment with CNBr the unnatural amino acid reporter group remains attached to the polypeptide in the position of the PEG chains. The polypeptide now devoid of the bulky polymer can then be more easily fragmented using proteolytic enzymes and sequenced by Edman degradation.

Numerous techniques other than N-terminal sequencing can be used to characterize the fragments created for peptide mapping. The simplest case involves fragmenting the conjugate and indirectly identifying the site of modification based on the absence of peptides seen with the native protein. For example, tryptic digestion followed by RP-HPLC was used to characterize the three positional isomers of mono-PEGylated EGF¹⁸³ and the sites of polymer modification for a TNFR : *Fc* chimeric protein.⁷¹ Peptide mapping can also be coupled with mass spectrometry techniques such as MALDI (matrix-assisted laser desorption/ionization) or ESI (electrospray ionization) to identify the distinct positional isomer(s) contained in complex mixtures of PEGylated proteins. Recently, peptide mapping coupled with MALDI mass detection was used to identify the distinct positional isomer(s) contained in previously fractionated complex mixtures of PEG-IFN- α -2b²¹⁶ and PEG-IFN- α -2a⁹⁴ and an unfractionated PEGylated growth hormone preparation⁷⁶ with a high degree of precision. In addition, the site(s) of polymer attachment of

PEGylated IL-2 were successfully determined using peptide mapping in combination with LC-MS (liquid chromatography with mass spectrometry detection).¹⁹³ Finally, capillary electrophoresis (CE) cab be used as a substitute to RP-HPLC separation and identification of the site(s) of polymer attachment.²⁰⁹

5.6.2.3 Molecular Weight/Molecular Size

The following section provides only a general overview of the unusual problems encountered when determining the molecular weight and/or molecular size PEG-modified proteins. For a comprehensive review of the structures, properties and characterization of natural and synthetic biomaterials, readers are encouraged to consult Chapter 1 in this book contributed by Professor Ghandehari and associates. Although the covalent attachment of PEG to a protein will naturally increase its molecular weight and size, the polymer's random coil structure frequently creates a situation where the increases in molecular size are much larger than the corresponding changes in molecular weight. This effect limits the accuracy of molecular weight results obtained from techniques based on hydrodynamic radius (e.g., gel-electrophoresis or size-exclusion chromatography). For example, when the molecular weight of a mono-PEGylated Fab' antibody fragment was estimated using SEC, the conjugate's apparent molecular weight was determined to be approximately 2000 kDa although its theoretical molecular weight was known to be around 149 kDa.¹¹⁴ In addition, it is commonly accepted that the mobility of PEGylated proteins in SDS-PAGE experiments is much slower than what would be expected from the known increase in molecular weight.²¹⁷

Because the PEG typically behaves 5 to 10 times larger than globular proteins of equivalent molecular weight, more rigorous analytical methods have been developed to characterize the molecular mass and/or size of PEGylated protein conjugates. A fairly simple quantitative method was developed that coupled the use of HPSEC with UV and RI detection to characterize the molecular size, polymer distribution, and polymer weight composition of PEG-proteins.²¹⁸ The method allows the prediction of molecular size based on viscosity radius and compensates for shape differences between PEG and protein structures. This approach also requires the construction of a universal calibration curve using globular protein and synthetic polymer standards of known molecular weight and polydispersity. Laser-light scattering has been used to determine the actual molecular weight and molecular size of PEGylated proteins when SDS-PAGE or other techniques have given false results.²¹⁹

Matrix assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry and electrospray-ionization (ESI) mass spectrometry are valuable tools for determining the molecular weight of PEG-protein conjugates. These so-called "soft" ionization techniques were created to minimize the fragmentation of labile macromolecules, permitting their (relatively) straightforward use in accurately determining the molecular

weight of PEGylated proteins. Both MALDI-TOF and ESI-MS are capable of yielding the complete picture of the components contained in complex mixtures based on molecular mass and can be used to characterize the molecular masses of any PEGylated protein species, irrespective of the conjugate's chemical properties or coupling method employed. However, both of these methods do not generate quantitative information because of the different extraction yields of each molecular weight compound from the matrix.

5.6.2.4 Conformational Analysis

Circular dichroism (CD) spectroscopy has been used to examine the effects of PEG modification on protein conformation.^{84,86,89,110,174,216,220,221} CD measures the difference in absorption between left- and right-handed circularly polarized light according to the following equation:

$$\Delta \varepsilon = \Delta A/cL = (A_L - A_R)/cL)$$

where A_L and A_R are the absorption of the left- and right-handed polarized light, respectively, c is the concentration of chromophores (e.g., peptide bonds, aromatic side chains, disulfides, etc.) in mole/dm³, L is the path length in cm, and $\Delta \varepsilon$ is the molar absorption difference in liter mole⁻¹ cm⁻¹. In the nearultraviolet range (250 to 320 nm) is mostly used to obtain global information on changes in tertiary structure upon differences in the conformationally dependent environments of the aromatic amino acid side chains (i.e., Trp, Tyr, and Phe) and cystinyl disulfide groups between native and PEG modified proteins. Quantum mechanically, near-UV CD originates from the coulombic interactions between asymmetrically positioned transition dipoles of independent chromophores within a protein molecule.²²² These interactions depend on the orientation of the dipole moments and therefore the configuration of the chromophores that lie within 4 to 5 Å of each other. The conformationally dependent environments of the aromatic amino acid side chains (i.e., Trp, Tyr, and Phe) and cystinyl disulfide groups are qualitatively measured. In the far-ultraviolet range (180 to 250 nm), the CD signal is dominated by $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ peptide bond transitions that essentially reflect the dihedral angles contained in a polypeptide. Because each of the secondary structural elements (e.g., α -helix, β -sheets, β -turn, random coil) contained in proteins and peptides have characteristic dihedral angles, far-UV CD can be used to monitor the secondary structure of both native and modified polypeptides. Usually the magnitudes of two negative minima at 208 nm (α -helix) and 223 nm $(\beta$ -sheet) are qualitatively evaluated. A semi-quantitative measure of the effects of PEG modification on protein structure involves using deconvolution programs like CDNN, which compare the far-ultraviolet dichroic spectra of the native species and its PEGylated derivative to a set of 33 basis spectra representing the major secondary structure elements commonly seen in peptides and proteins.²²³ Furthermore, the neural networks chosen for far-UV CD data analysis can account for both aromatic and disulfide contributions to a sample's dichroic intensity in the far-ultraviolet region. Both far- and near-UV CD provide fast and convenient method for observing the secondary and tertiary (i.e., conformation) structures of proteins and their PEG modified derivatives. Less frequently, Fourier-transform infrared spectroscopy (FTIR) and multi-dimensional NMR (e.g., NOESY, COSY, TOCSY, etc.) techniques are employed in conformational analysis of PEG-protein conjugates. It is important to note that even if the secondary and/or tertiary structure of the native and PEGylated protein species are similar, this does not ensure the conjugate will be fully functional because the polymer can sterically interfere with the intermolecular interactions necessary for activity.

5.6.2.5 Quantification of PEG-Proteins in Biological Samples

One of the most attractive results of PEGylation is the ability to reduce a protein's rate of clearance. Therefore, in many cases it is necessary to evaluate a conjugate's pharmacokinetics to determine the effects of PEGylation on therapeutic efficacy. Unlike small molecular drugs that are nowadays detected in biological samples using selective and sensitive techniques like tandem liquid chromatography-mass spectrometry (LCMS), PEGylated molecules cannot be sensitively detecting using such a technique because the conjugates are both polydisperse and heterogeneous. Therefore, other methods must be applied to sensitively measure conjugate levels in biological samples. Radioisotope labeling is frequently used to provide a practical and extremely sensitive means of detecting PEG modified proteins. Radiolabeled conjugates are normally used in a variety of in vitro and *in vivo* situations where very low levels of quantification or high levels of interfering substances are encountered. Probably the most common way to directly radiolabel a PEG-protein conjugate involves attaching a radioactive form of iodine (e.g., ¹²⁵I and ¹³¹I) to one or more tyrosine residues utilizing conventional techniques such as the ChloramineT and lactoperoxidase labeling protocols. In addition, ³⁵S-labeled Cys and Met residues can be directly incorporated into recombinant proteins by adding ³⁵S-labeled sulfate into the expression system¹⁰⁴ or radiolabeled forms of PEG (e.g., ¹³C or ³H) may be conjugated to proteins resulting in labeled species. Although the production of backbone labeled PEG is feasible, it involves several steps that are expensive and time consuming.²²⁴ A useful alternative is to prepare an activated PEG containing a radiolabeled amino acid spacer. However, all of these modes of (radiometric) detection depend on the assumption that the polymer and protein are irreversibly bound, therefore it may be necessary to employ dual labeling (i.e., different isotopic labels for protein and polymer portion of conjugate) or entirely different approaches to conjugate detection.

Immunoassays (e.g., ELISA, EIA, RIA, IRMA, etc.) are another popular way of measuring minute quantities of unlabeled PEG-protein conjugates in biological samples. These immunoassays all depend on the recognition of specific epitopes by an antibody and some sort of signal amplification (e.g., radiometric, enzymatic, etc.) for the sensitive quantification of conjugate species. Most of the immunoassays used in the place of direct radiolabeling to detect PEG-protein conjugates are based on antibodies that recognize proteinspecific epitopes. For example, protein-specific immunoassays (RIA, ELISA, and EIA) have been adapted or developed to quantify circulating levels of several PEG-protein conjugates, including PEGylated versions of insulin⁸⁴, EGF,⁸⁵ GH,⁷⁶ MGDF,²²⁵ leridistim,⁷⁴ nartograstim,⁷³ staphyloki-nase (SakSTAR) variants,²²⁶ lysostaphin,⁵⁶ IL-2,¹⁰⁵ and an anti-IL-8 $F(ab')_2$ fragment.¹¹⁵ An interesting alternative to the conventional immunoassay based approach was developed in which affinity chromatography coupled with an on-line immunochemical detection system was used to detect very low concentrations (<1 ng/mL) of PEG-G-CSF in rat serum.²²⁷ However, as mentioned in a previous section, the attachment of PEG to a protein commonly masks its antigenic epitopes from antibody recognition, severely limiting the sensitivity of immunoassay-based detection of conjugates in biological samples. This scenario provided the motivation for development of a sensitive ELISA assay to detect very low levels (i.e., 15 ng/mL) of PEGproteins based on the recognition of a PEG-specific (6 to 7 oxyethylene units) epitope.²²⁸ While the use of such polymer-specific immunoassays has yet to be realized beyond the scope of academic research, this approach has the potential to be a convenient and widely applicable way to quantify almost any conjugate whose polymer is of sufficient molecular weight to allow sufficient antibody cross-reactivity. Many potential techniques are available for detecting PEG-protein levels in biological samples (such as those obtained for pharmacokinetic analysis), each of which has associated benefits and shortcomings.

5.7 CLINICAL APPLICATION OF PEG-MODIFIED PROTEINS

The therapeutic value of proteins has been recognized for many years. However, most therapeutic proteins are unstable, immunogenic, and are rapidly cleared from the circulation. Consequently, high doses and frequent injections are often required to achieve therapeutic efficacy. The covalent modification of therapeutic proteins can alleviate many of the shortcomings inherent to protein drugs and the utility of PEGylation has been demonstrated in the clinic. The following section provides a brief summary describing each of the PEG-modified proteins currently approved for human use by the FDA (and other regulatory agencies).

In 1990, ADAGEN[®] (PEG-adenosine deaminase, Enzon, Inc., Bridgewater, NJ) became the first PEG-protein drug to receive approval by the FDA for human use. ADAGEN is used for enzyme replacement therapy for the treatment of severe combined immunodeficiency syndrome (SCIDS) associated with a deficiency of adenosine deaminase (ADA). In the absence of the enzyme ADA, the purine substrates adenosine, 2'-deoxyadenosine, and their metabolites are toxic to lymphocytes. In SCIDS patients the direct function of the enzyme is to correct for these metabolic abnormalities, and associated longterm improvements in immune function have been reported in these patients. However, bovine ADA is rapidly cleared from the plasma of experimental animals and the enzyme required PEG-modification to increase its half-life $(6.4\times)$ in rats.²²⁹ Therefore, the drug product was prepared in which bovine adenosine deaminase ($M_r = 40,000$ Da.) was modified with 11 to 17 mPEG-succinimidyl groups ($M_r = 5000$ Da.) via amide bond formation between the polymer and protein lysine groups, and supplied as a sterile solution for intramuscular injection.

The second PEG-modified protein drug approved by the FDA for human use was also a xenogenic therapeutic enzyme. In 1994, ONCASPAR[®] (PEGasparaginase, Enzon, Inc., Bridgewater, NJ) was approved for use in patients with acute lymphoblastic leukemia (ALL) who require L-asparaginase in their treatment regime and are hypersensitive to native forms of the enzyme. The native form of L-asparaginase derived from *Escherichia coli* has been used in leukemia treatment since the 1960s, but many patients developed hypersensitivity reactions to the highly immunogenic bacterial protein. Therefore, the native enzyme ($M_r = 135,000$ Da.) was modified with multiple strands of mPEG ($M_r = 5000$ Da.) via amide bond formation between the polymer and protein. The PEGylated enzyme was not only less immunogenic, but was cleared from the body about three times slower that its unmodified counterpart.

More recently, two different PEG-modified versions of interferon-alpha (IFN- α) were approved for use in treating patients with hepatitis C viral (HSV) infections. PEG-INTRON[®] (PEG-interferon- α -2b, Shering-Plough, Kenilworth, NJ) received FDA approval for human use in 2000. The native cytokine (IFN- α -2b, $M_r = 19,000$ Da) is reacted with an electrophilically activated polymer (SC-mPEG, $M_r = 12,000$ Da.) under slightly acidic (pH 6.5) conditions resulting in permanent (urethane) and reversible bonds (imidazolecarbamate, tyrosine/serine carbonate) between the polymer and protein. The final product is a mixture composed of >95% mono-PEGylated species. Interestingly, the majority (~48%) of these mono-substituted species is modified at a histidine residue (His³⁴), while the remaining 13 positional isomers are PEGylated at various lysines (K³¹, K¹²¹, K¹³⁴, K¹³³, K¹³¹, K¹⁶⁴, K^{83} , K^{112} , and K^{49}), the N-terminal cysteine (C¹), as well as serine (S¹⁶³), tyrosine (Y¹²⁹) and another histidine (H⁷) residue.²¹⁶ The circulation half-life of PEG-INTRON (48 to 72 hours) is approximately eight times that of the native protein permitting weekly subcutaneous dosing of the conjugate. Importantly, the continuous presence of the PEG-INTRON allows for increased antiviral efficacy compared to the thrice weekly dosing of the unmodified cytokine. PEGASYS[®] (PEG-interferon- α -2a, Hoffman La-Roche, Nutley, NJ) is the other PEGylated IFN- α product approved for treatment of HCV. This product was developed after research showed that fewer strands of PEG of higher molecular weight could be employed to enhance the cytokine's specific activity as well as its circulation half-life. It should be noted that IFN- α -2a and IFN- α -2b differ by only one amino acid residue. PEGASYS is synthesized by reacting the IFN- α -2a ($M_r = 19,000$ Da) with an electrophilically activated

branched polymer (mPEG₂-SPA, M_r =40,000 Da.) under slightly basic (pH 8.5) conditions, resulting in the formation of a hydrolytically stable amide bond between the polymer and protein. The reaction conditions were manipulated such that a relatively homogeneous mixture (94% modified at K³¹, K¹²¹, K¹³¹, or K¹³⁴) of mono-PEGylated (95 to 99%) species was generated.¹⁸⁵ Attachment of the large branched-polymer to IFN- α -2a substantially increased the conjugate's absorption half-life relative to either PEG-INTRON or the native cytokine. The result was a protracted action profile sufficient for once weekly dosing.

NEULASTA® (PEG-filgrastim, Amgen, Thousand Oaks, CA), the PEGconjugated derivative of recombinant human methionyl granulocyte-colony stimulating factor (G-CSF), is another example of a FDA approved PEGylated cytokine. Filgrastim ($M_r = 19$ kDa) is a water-soluble 175 amino acid protein expressed from Escherichia coli that is used to reduce the duration and severity of neutropenia (reduced white blood cell counts) associated with cancer chemotherapy. This cytokine and its PEGylated version act on hematopoietic cells by binding to specific surface receptors thereby stimulating their proliferation, differentiation, commitment, and end cell functional activation. In preparing PEG-filgrastim, the cytokine's N-terminal amino group selectively modified with an aldehvde-terminated mPEG is $(M_r = 20 \text{ kDa})$ via the reductive amination mechanism mentioned in a previous section. Because of the conjugate's increased molecular size $(M_r = 39 \text{ kDa})$ relative to the native protein, PEG-filgrastim has a reduced renal clearance and prolonged persistence in the circulation. A single 6 mg dose of NEULASTA was found to be as effective as 14 daily doses ($5\mu g/kg$) of the unmodified cytokine (NEUPOGEN[®], Amgen, Thousand Oaks, CA) in minimizing the number of days of severe neutropenia, depth of absolute neutrophil count (ANC), and time to ANC recovery after nadir.

SOMAVERT[®] (PEG-visomant, Pharmacia & Upjohn, Kalamazoo, MI) is a PEGylated human growth hormone (hGH) antagonist used in the treatment of acromegaly. The hGH antagonist B2036 (visomant) was modified with PEG to prolong its circulating half-life and lower its immunogenic potential.⁹¹ The unmodified protein $(M_r = 22 \text{ kDa})$ is an analog of hGH that has been structurally altered by genetic engineering to act as a GH receptor antagonist and possesses nine reactive amines (F¹, K³⁸, K⁴¹, K⁷⁰, K¹¹⁵, K¹²⁰, K¹⁴⁰, K¹⁴⁵, and K¹⁵⁸) available for modification with Pe.g., SOMAVERT[®] is prepared in such a way that an average of five electrophilically activated PEG chains $(M_r = 5 \text{ kDa})$ are attached predominantly to residues F¹, K³⁸, K¹²⁰, K¹⁴⁰, and K¹⁵⁸. PEG-visomant selectively binds to growth hormone (GH) receptors on cell surfaces, where it blocks the binding of endogenous GH and interferes with the receptor's signal transduction pathway. The inhibition of GH action results in decreased serum levels of insulin-like growth factor-1 (IGF-1) and other GH-responsive serum proteins resulting in the amelioration of complications associated with acromegaly. Similar to the case of PEGASYS, the absorption half-life of SOMAVERT is significantly increased. However, very large (10 to 20 mg) daily doses (equivalent to 20 to $40 \times$ normal levels of endogenous GH production) of PEG-visionant are required to treat acromegaly. Nonetheless, because SOMAVERT is the only therapy available for the treatment of patients with acromegaly that are resistant to somatostatin analogs, the need for daily injections does not deter from the drug's application.

The clinical development of ADAGEN, PEGASYS, PEG-INTRON, and NEULASTA was driven by the need to prolong the native protein's half-life, while ONCASPAR was developed primarily to decrease the xenogenic protein's immunogenicity. Interestingly, SOMAVERT was developed to both prolong the protein's circulating half-life and lower its immunogenic potential. In every case, the modification of therapeutic proteins with PEG resulted in drug products whose favorable properties served to increase their therapeutic efficacy, and in many cases created treatments for patient populations who did not respond to more conventional therapies.

5.8 CONCLUSIONS

Protein conjugation, cross-linking, and PEGylation are techniques that are growing rapidly within the biotechnology industries. Several biological applications of these technologies have been realized because of the numerous favorable properties imparted on proteins by PEGylation; the diverse classes of polymer structures, activating groups, and conjugation chemistries; and the well-developed methods for purifying and characterizing the resultant conjugates. It is expected that research and development in these areas will continue to thrive and the commercialization of products spawning from the biological applications of PEG technology will expand in the future. Of course, in order for this diverse technology to reach its full potential, certain aspects of it need to be researched further. For example, a more basic understanding of underlying reasons how protein PEGylation actually works would greatly aid in the creation of new therapeutic entities. Why does PEG modification prevent certain inter-molecular interactions (e.g., antibody-antigen), while allowing others (e.g., receptor-ligand) to occur? This frequently asked question lies at the heart of the technology, yet has not been systematically studied to date. In addition, the exact nature of the interactions between the polymer and protein deserve additional research. That is, what conformation(s) does the polymer adopt when conjugated to proteins and does this conformation depend on the physicochemical nature of the particular protein surface to which the polymer is attached? Furthermore, the effects of PEG modification on protein chemical stability have yet to be thoroughly addressed in the literature. Proteins are known to undergo many nonenzymatic reactions including deamidation, β -isomerization, oxidation, transamidation and polymerization, and disulfide exchange. It is also known that protein conformation can affect the rates of these decomposition reactions. Therefore, careful study of the effects of PEG modification on protein chemical stability will greatly enhance the utility of this technology. In light of the recent progress being made in the area of protein PEGylation, this technology seems to be gaining

acceptance from both the pharmaceutical industries and federal regulatory agencies as a valuable method of increasing the therapeutic efficacy of protein pharmaceuticals.

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6 Complement Activation by Injectable Colloidal Drug Carriers

Catherine Passirani and Jean-Pierre Benoît

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References

6.1 INTRODUCTION

Colloidal drug carriers are particularly useful for formulating new drugs derived from biotechnology (peptides, proteins, genes, and oligonucleotides) because they can provide protection from degradation in biological fluids and promote their penetration into cells. However, because the body is so well equipped to reject any intruding object, the materials which are to stand any chance of success within this hostile yet sensitive environment must be chosen very carefully. More particularly, attention has to be turned to the composition of the surface of colloidal drug carriers. Indeed, their clearance rate from the circulatory system is determined by their uptake by the mononuclear phagocytic system which depends in turn on their physico-chemical surface characteristics. In order to enhance circulation time, steric protection of various nanoparticulate drug carriers can be achieved by grafting hydrophilic and flexible polymers to their surface. The protective effect of these polymers may be interpreted in terms of statistical behavior of macromolecules in solution, where polymer flexibility plays a key role. Because of the importance of the complement system in opsonization, it plays a major, but probably not exclusive, role in inducing a fast phagocytosis of colloidal drug carriers. In the search for injectable, biocompatible, and long-circulating systems, the evaluation of their interactions with complement represents a good preliminary test predictive of the *in vivo* fate of the colloidal drug carriers after intravenous administration.

6.2 THE COMPLEMENT SYSTEM

In "complement" to the action of antibodies, the complement system plays an essential role in host defense against infectious agents and in the inflammatory

process. Part of both intrinsic defenses and acquired defenses, it consists of about 20 plasma proteins that function either as enzymes or as binding proteins. In addition to these plasma proteins, the complement system includes multiple distinct cell-surface receptors that exhibit specificity for the physiological fragments of complement proteins and occur on inflammatory cells and cells of the immune system. There are also several regulatory membrane proteins that function to prevent autologous complement activation and protect host cells from accidental complement attack.¹ The presence of microorganisms, immune complexes or any intruding object, triggers activation of the complement cascade, which involves a deposition of complement proteins on the foreign surface, with the assembly of cytolytic complexes, the enhancement of phagocytosis and the initiation of the inflammatory response.

The complement system can be activated by three different pathways, known as the classical, lectin and alternative pathways. The first pathway, known as the classical pathway for historical reasons (it was discovered first), is activated by antibody opsonization of pathogens (binding of antibodies to specific epitopes of surface proteins, e.g., antigens) and is therefore a part of adaptive immunity. The lectin pathway, sometimes called the lytic pathway, is initiated by complement receptors comprised of circulating serum lectins (lectins are proteins that bind to sugar moieties), which bind pathogen surface molecules containing mannose residues (e.g., mannan). Finally, since its activation may occur in the absence of antibodies, the alternative pathway is spontaneously activated at the first encounter of a pathogen. Its activation during the contact of plasma with artificial materials stresses the role of this pathway in biocompatibility and recognition of foreign substances such as colloidal drug carriers.

Each of the three pathways differs in the proteins involved in initiation, but converges at the point of amplification and the assembly of terminal lytic components (Figure 6.1).

6.2.1 ACTIVATION PATHWAYS

Table 6.1 gives the list and the function of different proteins constitutive of the complement system, the central component of complement being the abundant plasma protein C3. Complement activation occurs with each component sequentially: the first component cleaves specifically the second one into two fragments, one of these fragments cleaves the third component into two fragments, and so on. Complement has the special property of being able to deposit on particle surfaces after the activation of specific bonds. The main fragments of C2, C3, C4, C5, and B (C2a, C3b, C4b, C5b, and Bb) obtained after activation present, for a very short time (<10 ms), a binding-site for membranes (in the case of C3b, C4b, and C5b) or for protein acceptors already fixed to a membrane (C3b for B, C4b for C2a). If no acceptor is encountered, the molecule stays in the circulation and is rapidly deactivated. This phenomenon limits the development of activation to the microenvironment



FIGURE 6.1 The main pathways and components of the complement activation system.

TABLE 6.1Name and function of the different proteins constitutive of the complementsystem

Function	Proteins
Activation	Clq, Clr,* Cls,* C4, C2,* MASP1,2,* C3,
	C_{3D} -like, B,* D*
Membrane-binding opsonins	C3b, C4b, MBP
Inflammation mediators	C3a, C4a, C5a
Membrane attack	C5b, C6, C7, C8, C9
Complement receptors	CR1, CR2, CR3, CR4, C1qR
Complement regulatory proteins	C1 inh, C4bp, I,* CR1, MCP, DAF, H, P, S, CD59
*Activating enzymes	

at the site of action. The sequence of component interactions of the complement system is discussed in the following sections.

6.2.1.1 The Classical Complement Pathway

The classical pathway of complement activation is a mediator of the specific antibody response. It is the binding of a specific part of the antibody molecule

to the C1 component that initiates this pathway. This initial enzyme, C1, complex formed by the association of two reversibly interacting subunits C1q and (C1rC1s)₂, is able, through a calcium-dependent mechanism, to bind to immune complexes (specifically IgM and IgG).² A minimal fixation density of two IgG is necessary to overcome the controlling action of C1 inhibitor (C1 inh) whose role is to limit auto-activation.³ Then, the homologous C1r and C1s sub-components are converted into catalytically active species which cleave C4 and C2 into their fragments a and b. The C2a fragment becomes firmly associated with C4b via a magnesium-dependent reaction, and hence the C3 convertase, C4b2a, is generated. This enzyme, whose half-life does not exceed 10 minutes at 37°C, is now able to cleave the next component of the cascade, C3, into a small fragment C3a and a larger one, C3b. C3a appears to be important in many inflammatory responses, and C3b, which becomes covalently bound to the invading surface, appears to be of great importance in the process of opsonization.

C3 and C4 possess a thioester group protected in a hydrophobic pocket (Figure 6.2a). When cleaved, a conformational change allows the exposition of this active site which becomes accessible to nucleophilic molecules. This labile site can form covalent binding with a hydroxyl or amine group of a surface. In the absence of such acceptor sites for C4b or C3b, the labile binding site is no longer available (by hydrolysis, for instance) and they become incapable of furthering the complement cascade.⁴

6.2.1.2 The Lectin Complement Pathway

The lectin pathway is initiated by binding of mannan binding protein (MBP) and MBP-associated serine protease (MASP), which is present as a proenzyme. MBP reacts to N-acetylglucosamine residues on surface proteins of pathogens, thus activating MASP. The activated MASP then proteolytically cleaves C4 and C2. C2 is cleaved to form C2b and C2a. As in the classical pathway, the C3 convertase C4b2a is generated.

6.2.1.3 The Alternative Complement Pathway

The alternative pathway constitutes a humoral component of natural defense against infections, which can operate without antibodies. The six proteins C3, B, D, H, I and P (Table 6.1) together perform the functions of initiation, recognition, activation, and regulation of this pathway which results in the formation of activator-bound C3 convertase.

C3 has been shown to contain a thioester bond (Figure 6.2a). At a background level, the spontaneous slow hydrolysis of this thioester converts inactive native C3 to a functionally active C3b-like molecule⁵ (Figure 6.2b) responsible for the formation of the initial C3 convertase of the alternative pathway, upon the Mg²⁺-dependent binding of factors B and D. This resulting fluid-phase C3 convertase is able to cleave C3 to C3b. Cleavage of C3 generates



FIGURE 6.2 Schematic representation of (a) C3 activation (C3, C3a, and C3b can be replaced by C4, C4a, and C4b, respectively) and (b) C3b-like molecule.

a metastable binding site (Figure 6.2a) which promotes the covalent binding of C3b to a variety of cell surfaces, foreign as well as autologous. These reactions are positively regulated by properdin (P), whose function is to bind itself to cell-bound C3b and to stabilize the C3/C5 convertase⁶ and is negatively regulated by factors H and I, thus preventing the formation of convertase in fluid-phase and on autologous cells (Table 6.2).

Surface-bound C3b, the product of the reaction catalyzed by the C3 convertase, forms a subunit of the convertase itself (C3bBb), which is the cause of positive feedback: each newly produced C3b molecule has the potential to form the surface-bound convertase together with B, D, and Mg^{2+} , and thus to produce more C3b and more convertase. This constitutes the driving force of amplification of the alternative pathway, a property lacking in the classical pathway.

Discrimination between activating and non-activating surfaces in the blood depends on the outcome of competition between B and H for binding to surface-fixed C3b molecules and on the nature of this surface. Subtle conformational changes imposed on bound C3b by its microenvironment can be responsible for the B vs. H binding preference.⁷ When C3b binds to H, it is subsequently cleaved to C3bi (inactive) and the dissociation of C3bBb is accelerated. Fluid-phase regulators such as factors H and I in association with host cell-associated regulatory proteins (decay accelerating factor (DAF),
TABLE 6.2				
Regulatory p	roteins of	the com	plement sy	ystem

Proteins	Function
C1 inhibitor (C1 inh) C4 hinding protein (C4bp)	Inhibition of C1 protease
Factor I Complement receptor 1 (CR1) Decay accelerating factor (DAF) Membrane cofactor protein (MCP)	Protease, inactivates C3b and C4b Inhibits both C3 convertase formation Inhibits both C3 convertase formation
Factor H Properdin P	Inhibits alternative pathway C3 convertase, cofactor for Factor I Stabilizes the C3/C5 convertases
Protein S Membrane inhibitor of reactive lysis (CD59)	Inhibits membrane attack complex formation Inhibits membrane attack complex

membrane cofactor protein (MCP), complement receptor 1 (CR1)⁸ constitute a very efficient scavenger system for C3b and C3b-like molecules (Table 6.2). C3b degradation in its different fragments after binding to a nonactivating surface is represented in Figure 6.3.

6.2.2 THE MEMBRANE ATTACK COMPLEX

The three activation pathways lead to the formation of the target surfacebound C3 convertases (C4b2a and C3bBb). Provided an additional C3b molecule is available in close proximity, they generates C5 convertases (C4b2a3b and C3bBb3b). This results in the cleavage and activation of C5 into C5a and C5b. C5a diffuses in the fluid phase and plays the role of an inflammatory mediator while C5b interacts with C6, C7, C8, and C9 to form the membrane attack complex (MAC or C5b-9).9 Through its metastable membrane binding site, the forming MAC binds firmly to target membranes owing to hydrophobic interactions with their lipid bilayers. Then, the unfolding and oligomerization or polymerization of C9 cause the formation of transmembrane channels, allowing bidirectional flow of ions and macromolecules, thus leading to the osmotic lysis of the cell. Complement-mediated lysis can occur in many kinds of cells: erythrocytes, platelets, bacteria, viruses possessing a lipoprotein envelope, and lymphocytes. Colloidal drug carrier systems are potential targets too. For instance, the formation of a pore-like membrane attack complex in liposomes of around 10 nm diameter, is known to facilitate drug release after activation of the complement cascade.¹⁰

MAC can also form in solution the terminal complement complex (TCC). It should be noted that TCC formation and MAC assembly are regulated by the S protein of plasma. Additionally, homologous restriction factors of host



FIGURE 6.3 Schematic representation of C3b degradation by factor I after binding to a non-activating surface.

cell membranes like the membrane inhibitor of reactive lysis (CD59) (the most studied one) are regulators that function to restrict the assembly or insertion of the membrane attack complex (Table 6.1 and Table 6.2).

6.2.3 THE BIOLOGICAL CONSEQUENCES

The continuous generation of C3b-like molecules and the fluid-phase C3 convertase results in the indiscriminate deposition of C3b on foreign particles as well as on autologous cells. Although there is a continual non-specific initiation of the cascade, host cells generally restrict activation and amplification and are thus protected from complement-mediated damage. This restriction is mediated by specific regulators of the complement cascade as already described for each pathway. These regulatory proteins are compared in Table 6.2. When complement activation is really triggered, the three major consequences are: (1) direct cytolysis by MAC, as previously described, (2) enhanced clearance by phagocytes, and (3) initiation of the inflammatory response, even in the pre-immune phase where specific antibodies and lymphocytes are not available.

6.2.3.1 Cellular Interaction

Activation and amplification of the complement cascade lead to the deposition of very large amounts of C3b on activator surfaces. For example, an

Escherichia coli strain was shown to bind approximately 30,000 molecules of C3b per cell using purified complement components in the absence of antibodies.¹¹ The high molecular weight fragments of C3 and C4 (C3b and C4b) bound to cell membranes, bacteria or foreign particles, are recognized by various cells that have receptors, such as phagocytic cells (macrophages, monocytes, and polymorphonuclear leucocytes), B lymphocytes, neutrophils, erythrocytes, and platelets.¹² Particles that are coated with C3 and C4 fragments can, also, bind to erythrocytes via complement receptors such as CR1 and are then transported to the liver where macrophages remove them from the erythrocyte surface. Therefore, C3b and C4b molecules serve as a bridge between a complex or a target cell and the responding cell having a receptor for C3b or C4b. Since B lymphocytes have receptors for C3b and C4b, it is postulated that bridging brings antigens in direct contact with the antibody-forming cells and that, therefore, bound complement components may play a role in the induction of an immune response.¹³

The coating of organisms by molecules that speed up phagocytosis, is termed opsonization from the Greek "preparing a meal". Major opsonins include fixed C3 and C4 fragments, specific immunoglobulins, apolipoproteins, fibronectin, and coagulation factors. Liver, spleen, and bone marrow function as major sites of clearance of opsonized particles.

6.2.3.2 Role of Complement System in Inflammation Process

Activation of the complement cascade leads to the fragmentation of C3, C4, and C5 into low-molecular-weight hormone-like peptides, C3a, C4a, and C5a with anaphylatoxin activity. C5a also functions as a chemoattractant, inducing the migration of leukocytes and macrophages into an area of complement activation. C3a and C5a also stimulate monocytes and macrophages to synthesize and secrete interleukin-1 (IL-1).¹⁴ These anaphylatoxins induce smooth muscle contraction and enhance vascular permeability. They bind to specific receptors and induce the release of vasoactive amines such as histamine from mast cells and basophils, and lysosomal enzymes release from granulocytes (particularly C3a and C5a).^{15,16}

6.2.4 COMPLEMENT SYSTEM AND BIOMATERIALS

A wide variety of materials is being increasingly used in pharmaceutical and medical practice for the treatment of patients in which the materials come into direct and often sustained contact with body tissues. As in Williams' definition,¹⁷ we will use the term "biomaterial" for any nonviable material, natural or artificial, intended to interact with biological systems. The living materials such as cells, tissues, etc., will not be described.

Since activation may occur in the absence of antibodies, the alternative pathway is the complement pathway that is usually triggered when plasma is exposed to biomaterials. As shown previously, a spontaneous initiation of activation, remaining at the background level in the fluid phase, is due to the fact that a very small proportion of C3 exposes a thioester site, the so-called C3b-like molecule. This spontaneous activation is controlled in solution by the regulatory proteins H and I so that this phenomenon remains at background level. The nature of the resulting surface binding of C3b is crucial to determine whether or not the biomaterial will be an alternative pathway complement activator. In the presence of both factors B and H in plasma, both factors will compete for bound C3b. The outcome of the competition is determined by the surface type. If the C3b is protected from factor H, then preference is given to factor B; the convertase complex C3bBb is formed and the biomaterial becomes an activator capable of producing more active C3b cleavage products. If the C3b is accessible to factor H, then the complex C3bH allows cleavage of C3b to C3bi by factor I and the alternative pathway is deactivated.¹⁸

However, the activation of complement by artificial materials through the classical pathway has been reported in the case of some crystalline polymer surfaces or some complexes between positively and negatively charged molecules.¹⁹ Other examples of classical pathway activation through antibody-independent processes^{20–23} as well as alternative pathway activation by antibody-mediated processes^{24,25} are not unusual. Copolymers such as Cremophor EL[®], as well as poloxamer and poloxamine (Figure 6.4), can activate the complement system through both the alternative and classical pathways.^{26,27} In addition, some unknown or even unexpected antibodies can intervene. The binding of naturally occurring and abundant anticholesterol antibodies²⁸ to the hydroxyl-rich surface of certain polymeric materials is suspected since the epitope they recognize contains a hydroxyl group, explaining the activation of the classical pathway by liposomes (see Section 4.2.1), for example.

One well-known biochemical characteristic that determines the capacity of a particle surface to activate the human alternative complement pathway is a relative deficiency of sialic acid. The conversion of a nonactivating cell, the sheep erythrocyte, into an activating cell by the removal of sialic acid residues has been observed in early studies.²⁹ Additional cell-surface constituents such



FIGURE 6.4 General structure of (a) poloxamers and (b) poloxamines.

TABLE 6.3Examples of polyanions interacting with the complement system

Activators	Nonactivators	
Dextran (>60,000)	Dextran (<60,000)	
Dextran sulfate (>10,000)	Dextran sulfate (<10,000)	
Sephadex particles (crosslinked form of dextran)	Sialic acid	
	Heparin	
Cellulosic membranes	Heparan sulfate	
Zymosan particles	Fucan, fucoidan	

as heparan sulfate can also modulate the capacity of a particle to activate the alternative pathway.³⁰ At present, the most studied biomaterials have been polysaccharides, and among them dextran and heparin. They contain repeating subunits and look like surface components of complex activators or nonactivators such as bacterial cells. Some examples of polyanions which will be described here are presented in Table 6.3. The available information concerning their action is still incomplete since, even in the case of well-studied molecules, only partial aspects have been covered because of the complexity of the possible interactions.

6.2.4.1 Dextran and its Derivatives

Dextran is a hydrophilic polysaccharide made up of polymers of α -D-glucose units (Figure 6.5a).³¹ Linear dextrans are produced by the fermentation of sucrose with a strain of the bacterium *Leuconostoc mesenteroides* followed by hydrolysis and fractionation to provide dextrans with different average molecular weights. The alternative pathway is activated by dextrans with molecular weights of 60,000 Da and more. The sulfation of dextrans can modify their potential activity towards complement. Dextran sulfates of low molecular weight (Figure 6.5b) inhibit the alternative pathway, while molecules of 10,000 Da and larger have the potential of initiating the alternative pathway in serum (Table 6.3).³²

Sephadex[®], which is a cross-linked dextran, resembling cellulose in its basic chemical structure, constitutes a good model to study the interactions between complement and polysaccharidic or cellulosic surfaces. The activating capacity of Sephadex[®] is believed to depend on the availability of a large number of reactive hydroxyl groups that can form ester linkages with C3b and on the ability of the insoluble polymer to promote the formation of a protected surface-bound amplification C3 convertase. In addition, the repeating nature of cellulosic or polysaccharidic structures means that many potential sites for active convertase exist on the same surface. It is probably important that local amplification of enzymatic activity can occur in order that the material behave



FIGURE 6.5 Structure of (a) dextran, (b) dextran sulphate and (c) heparin.

as an activator.¹⁸ These different observations can explain why complement activation invariably occurs during haemodialysis performed with cellulosic membranes.³³

Different substitutions of hydroxyl groups (OH) in Sephadex[®] by carboxymethyl, benzylamide sulfonate, diethylaminoethyl, and other groups, led to the conclusion that the reduction of the availability of OH groups actually decreases complement activation but the relationship is probably not linear.^{33–36} A simpler polystyrene model on which either isolated hydroxymethyl or sulfonate groups or both were present permitted to demonstrate that the presence of sulfonate groups in the immediate vicinity of OH groups on the same backbone modulates the activating function brought by the hydroxyl groups.³⁶

In addition to the action of hydroxyl groups on the alternative pathway, specific anti-dextran antibodies may enhance Sephadex[®]-induced complement activation.^{37,38} The same antibodies are involved in dextran-induced immediate and dose-dependent C3 activation during cardiopulmonary bypass via the classical pathway.³⁹

6.2.4.2 Heparin and its Derivatives

The alternative complement pathway is capable of discriminating human cells and tissues from a wide variety of potential pathogens. The attachment of complement component C3b to activator surfaces restricts inactivation of C3b by factors H and I. This restriction is reversed by certain soluble polyanions (e.g., sialoglycopeptides, heparin, or dextran sulfate) (Table 6.3) that mimic the effects of sialic acid and glycosaminoglycans on human cells and tissues. These fluid-phase polyanions enhance the binding of factor H to C3b that is attached to activating particles, indicating that the effect results from an increased affinity between C3b and factor H.⁴⁰ Factor H is composed of a linear array of 20 homologous, short, consensus-repeat domains with many functional sites. It has been recently demonstrated that three of these sites are involved in binding C3b and regulating complement activation; others bind to sialic acid and/or heparin and are responsible for host recognition.⁴¹ The interactions of the polyanion binding site on factor H with glycosaminoglycans depends upon the number, orientation and polymeric arrangement of sulfate groups and suggest that most, but not all, sulfated glycosaminoglycans participate in the protection of host tissues from complement damage by promoting the deactivation of tissue-bound C3b.⁴²

Heparin is a natural polydisperse sulfated copolymer of glucosamine and uronic acid (Figure 6.5c). Inhibition of complement activation by heparin is demonstrated at various levels of the cascade,⁴³ in particular at the cell lysis level via the alternative pathway. The inhibitory capacity of heparin on the formation of the alternative pathway convertase requires N-substitution of the glucosamine and O-sulfation of the molecule.⁴⁴ Among a wide range of biological activities, heparin catalyzes the complex formation between anti-thrombin III and the proteases of the coagulation system but the independence of the anti-coagulant and anti-complementary sites on the molecule is definitively established.⁴⁵

There are numerous examples of the inhibition of complement activation by heparin. The covalent coupling of heparin to activating particles of zymosan,³⁰ or treating polystyrene microtiter plates with heparin suppress, for example, the capacity of the different surfaces to activate the alternative pathway.^{30,46,47} Intraocular lenses made from poly(methyl methacrylate) (PMMA) activate the complement system via the alternative pathway. The heparinization of PMMA eliminates the ability of this material to activate the complement cascade.^{48,49} Similarly, after modification with end-pointimmobilized heparin of surfaces like poly(vinyl chloride), silicone and a medical-grade aliphatic polyurethane, activation of the complement system is completely eliminated for all materials, both on the surface and in the fluidphase.⁵⁰ One clinical context in which heparin has found a secure niche is in the coating of extracorporeal circuits in cardiopulmonary bypass, renal dialysis and the like.51,52 Heparin coating markedly reduces activation of the complement and the coagulation systems in the circuit, reducing many of the inflammatory sequels of the procedure.⁵³ The capacity of surface-bound heparin to inhibit alternative pathway activation in vitro³⁰ as well as *in vivo*⁵⁴ is therefore of particular interest for the design of biocompatible surfaces.

The inhibitory effect of heparin on complement activation may be mimicked by synthetic dextrans substituted with sulfonated and carboxylic groups.⁵⁵ Other natural sulfated polysaccharides such as fucans or fucoidan, were found to be potent inhibitors of human complement too, and have been studied recently.^{56–58} Inhibitors might also be found among the endogenous regulatory proteins of complement that block the enzymes that activate C3 and C5. Of these proteins, complement receptor type 1 (CR1) has the highest inhibitory potential, but its restriction to a few cell types limits its function

in vivo. This limitation was overcome by the recombinant soluble human CR1, sCR1, which has proved to be a potent inhibitor of complement activation by both pathways.⁵⁹

6.3 INJECTABLE COLLOIDAL DRUG CARRIERS

To exert efficiently its activity without toxic effects, a drug must reach its pharmacological site(s) of action within the body. One of the most promising approaches to achieve site specific delivery is the use of a carrier (Figure 6.6). Colloidal drug carriers such as liposomes and nanoparticles can be used to alter the normal physiological distribution of a new or established drug and direct it according to the physico-chemical properties of the carrier, rather than that of the drug itself. The concept of drug targeting, suggested by Paul Ehrlich almost a century ago, considering a hypothetical "magic bullet" able to recognize and bind the target and to provide a therapeutic effect, is still the one required to solve the delivery problems posed by new classes of active molecules, such as peptides, proteins, oligonucleotides and genes.



FIGURE 6.6 Schematic representation of different colloidal drug carriers. Nanoparticles are polymer or lipid particles constituting nanospheres or nanocapsules. Micelles are self-assembled block copolymers that form a hydrophilic layer and an inner hydrophobic core. Liposomes are lipid structures forming uni- or multi-lamellar vesicles. Dendrimers are monodispersed symmetric macromolecules with a large number of reactive end groups. Ferrofluids are colloidal solutions of iron-oxide, magnetic nanoparticles surrounded by a polymer or dextran layer.

6.3.1 DISTRIBUTION OF COLLOIDAL DRUG CARRIERS ADMINISTERED INTRAVENOUSLY

The expected potential applications of intravenous colloidal drug carriers are: the concentration of drugs in accessible sites, the rerouting of drugs away from toxicity sites and an increase of circulation time of labile or rapidly eliminated drugs such as peptides and proteins.⁶⁰

6.3.1.1 Capture by Mononuclear Phagocytic System

The main limit of intravenous administration is that colloidal drug carriers are rapidly recognized by the macrophages of the Mononuclear Phagocytic System (MPS), particularly Kupffer cells in the liver, and are removed within seconds from the blood stream, because of opsonization by plasma proteins. As previously shown, the opsonization process is the adsorption of protein entities capable of interacting with specific plasma membrane receptors on monocytes and various subsets of tissue macrophages, thus promoting particle recognition by these cells.

Three physico-chemical properties of the colloidal carrier surface can affect the opsonization process: charge, hydrophilicity/hydrophobicity, and size. These influence interaction forces that govern adhesion of the particle to the phagocytic cell. Despite contradictory studies regarding charge effect, it is commonly admitted that hydrophobic and negatively charged particles increase the clearance of colloidal drug carriers from the circulation. Hydrophilic particles with neutral surfaces seem the most appropriate with regard to blood persistence. For example, early studies with liposomes demonstrated that small neutral unilamellar vesicles of saturated phospholipids and cholesterol have a longer circulation time in rats (half-lives up to 20 h) than their anionic counterparts (half-lives less than 1 h).⁶¹ Together with surface characteristics, the size of the colloidal drug carrier can also determine biological fate. Indeed, in the ideal range of 70 to 200 nm, the surface curvature of particles may affect the extent and/or the type of protein or opsonin adsorption, which play a critical role in complement activation (see also Section 6.4.2 and Figure 6.13).⁶²

Some authors suggest that the rate of clearance of particles from the blood by hepatic macrophages is regulated by a balance between opsonins and dysopsonins.^{63–66} Dysopsonins, like serum albumin, whose role is to protect the phagocytic cells from being destroyed by the excessive ingestion of particles, would be able to replace opsonins at the particle surface. The identification of these blood proteins and their mode of action could provide another way of enhancing particle circulation time.⁶²

Besides the opsonization process, one study has shown the ability of stimulated or activated macrophages to rapidly recognize and ingest longcirculating particles by an opsonic-independent process.⁶⁷ Under normal physiological conditions, the putative macrophage receptors are either down regulated or exocytosed from the cell surface and circulate in soluble form in the blood.⁶⁸ On the contrary, in clinical situations, enhanced macrophage activity can be observed and this opsonic-independent capture by MPS could be of particular relevance. In agreement with these studies, some reports have demonstrated that long-circulating liposomes are prone to phagocytosis by macrophages located at pathological sites with leaky vasculature, such as infection sites and solid tumors.⁶⁹ In addition to vesicle recognition by stimulated or activated macrophages, the elevated local concentration of phospholipases may further enhance liposome internalization by macrophages.⁷⁰ The head groups of the phospholipids on the liposomes can also undergo direct recognition of any of these groups by charge- or head group-specific receptors on phagocytes in vitro and *in vivo*.¹⁰

Since colloidal drug carriers are naturally concentrated within macrophages, various lipid and polymeric carriers are in extensive use for targeting these cells.⁷¹ For example, the liposomal preparation of amphotericin B is marketed world-wide for the treatment of visceral leishmaniasis. Present drug candidates that have been tested for transfer to macrophages include azidodeoxythymidine (AZT), dideoxycytidine, dideoxyinosine and antisense oligodeoxynucleotides. Immunomodulators such as interleukin-2, interferon, muramyl dipeptide (MDP) and their lipophilic derivatives are very good candidates and are extensively reviewed by Ahsan et al.⁷¹ For example, taking advantage of their MPS uptake, nanocapsules of muramyltripeptide cholesterol proved to be more efficient than the free drug in activating macrophages and stimulating their innate defense functions against liver tumor cells.⁷² In the case of protein antigens, their encapsulation in liposomes increases capture rates by antigen-presenting cells such as macrophages, thanks to the potential of liposomes as immunological adjuvants.⁷³ In an alternative strategy, immunogenic peptides have been coupled to the surface in order to activate B and T cell clones directly.⁷⁴ Liposomes have also been used as carriers in DNA vaccines.75

6.3.1.2 Extravasation through Blood Vessel Endothelium

If opsonization and MPS capture of colloidal drug carriers are avoided (see next section), what is the fate of these systems? It will depend on their distribution within the organism via general vasculature, given that the structure of blood vessel endothelium significantly varies from organ to organ (Figure 6.7). Extravasation of colloidal carrier is possible only in organs with discontinuous and fenestrated endothelium, where transfer may occur via direct diffusion through wide intercellular gaps. Particles larger than 50 nm were never reported to extravasate significantly, except in organs with specialized endothelium such as the liver, the spleen and bone marrow. Even in these organs, the size of the gaps between endothelial cells (approximately 100 nm) means that only the smallest particles can penetrate the tissue. However, the permeability of vascular endothelium may be temporarily increased by numerous factors associated to pathological processes such as complement activation.⁷⁶ Colloidal carriers may extravasate in a variety of



FIGURE 6.7 Different structures of blood vessel endothelium. (a) Capillaries with continuous endothelium are the most common type. The endothelial cells form a continuous internal lining without any intercellular or intracytoplasmic defects. (b) In fenestrated endothelium, the endothelial cells are pierced by pores (fenestrations) that provide channels across the capillary wall. Examples of fenestrated capillaries can be found in the gastrointestinal mucosa, in certain endocrine glands such as the hypophysis and the thyroid gland and in the renal glomeruli. (c) Discontinuous endothelium (sinusoidal capillaries or sinusoids) forms large, irregularly shaped and thin-walled blood vessels lined by endothelial cells and specialised phagocytic cells as Kupffer cells of the liver. Unusually wide gaps are present between the endothelial cells that permit leakage of material into and out of these vessels. There may be a partial or complete absence of the basal lamina underlying the endothelium. Sinusoids can also be identified in spleen and bone marrow.

pathologies with abnormally high vascular permeability, such as inflamed tissues and tumors. Indeed, most solid tumors possess unique pathophysiological characteristics that are not observed in normal tissues or organs, such as extensive angiogenesis and hence hypervasculature, defective vascular architecture, impaired lymphatic drainage/recovery system, and a greatly increased production of a number of permeability mediators.⁷⁷ The phenomenon now known as the "enhanced permeability and retention effect" (EPR) has been observed to be universal in solid tumors and, primarily, its role is to sustain an adequate supply of nutrients and oxygen for rapid tumor growth. The EPR effect also provides a great opportunity for more selective targeting of colloidal drug carriers to the tumor (Figure 6.8).

Since the "cut off" size of permeabilized vasculature can vary from case to case, the size of a drug carrying particle may be used to control the efficacy of such spontaneous or "passive" drug targeting. Liposomes of diameters greater



FIGURE 6.8 Enhanced permeation retention (EPR) effect. As growing tumours establish their own blood supply, they develop a distinct neovasculature whose blood vessels are frequently hyperpermeable ("leaky") to circulating macromolecules or colloidal drug carriers. In addition to this enhanced permeability, tumour tissue often has limited lymphatic drainage, so particles can be trapped and concentrated in tumours.

than 300 nm, for example, accumulate significantly in the spleen, as a result of mechanical filtration, followed by eventual phagocytosis in red pulp macrophages.^{78,79} Therefore, particles and their aggregates should be small enough so that they are not removed from circulation by simple filtration. The fact that the size of a particle may change substantially upon introduction into a protein containing a medium such as plasma also has to be considered. On the other hand, due to the possible penetration of very small particles through fenestrae in the endothelial lining of the liver, and to the fact that they can also leave the systemic circulation through the permeable vascular endothelium in lymph nodes,⁸⁰ a lower size limit should be taken into account, limiting a relatively narrow size range of 70–200 nm for the ideal colloidal drug carrier.^{81,82}

It has also been shown that liposomes that are sufficiently small to pass through the approximately 100 nm pores in the sinusoidal endothelium of the liver, can have access to the hepatocytes (Figure 6.7c) and are actively internalized and processed by this cell type. It was demonstrated a long time ago that the smaller the liposomes (below 100 nm in diameter), the larger the contribution of hepatocytes in total hepatic uptake.⁸³ Even highly deformable vesicles of up to 400 nm in diameter can also reach hepatocytes after intravenous injection by a process of "extrusion" through endothelial fenestrae.⁸⁴ However, although size is a determining factor, lipid composition is also, in this case, an important parameter to take into account, including charge, rigidity and headgroup composition.⁸⁵

6.3.2 Systems Avoiding Uptake by Phagocytic Cells

The accumulation of these "first-generation" drug carrier systems in the MPS organs, namely the liver, the spleen, the lungs, bone marrow and lymph nodes,

greatly limits their potential. As a result, a great deal of work has been focused on the development of long-circulating carriers also called stealth carriers. Such systems have been recently developed in order to improve the delivery of protein or nucleic acids.^{86–93} The expected advantages are a maintained level of a pharmaceutical agent in blood for extended time intervals, an accumulation (or passive targeting) in pathological sites with leaky vasculature such as tumors, inflammation and infracted areas, and a slow release of the drug in these areas. Active targeting by specific ligand-modified carriers (see Section 6.3.2.3) also requires their invisibility to macrophages and a prolonged half-life in the blood compartment. These properties are usually achieved by chemical modification of the colloidal drug carrier surface with synthetic or natural polymers.

6.3.2.1 PEGylated Systems

6.3.2.1.1 Protein–Surface Interactions in the Presence of Poly(ethylene glycol)

Poly(ethylene glycol) (PEG), also called poly(ethylene oxide) (PEO or POE), is a homogenous, non-toxic and water-soluble polymer represented by the formula $HO(CH_2CH_2O)_nH$ (Figure 6.4). Its polyether backbone is inert in the biological environment, as well as in most chemical reaction conditions such as in chemical modification and/or conjugation reactions. The presence of two terminal hydroxyl groups allows PEG molecules to be attached to various species by covalent coupling.⁹⁴

PEG has minimal interfacial free energy with water and hence high hydrophilicity, high surface mobility, and steric stabilization effects. In addition, its unique solution properties and molecular conformations in water make PEG a particularly effective polymer for the prevention of protein adsorption by forming a dense distribution of probable conformations, the so-called "conformational cloud" (Figure 6.9).^{95,96} The influence of molecular weight, surface density and conformation of PEG affect dramatically the protein adsorption pattern. In the model developed by Jeon et al.⁹⁶ it is shown that a brush (one chain end attached-PEG or "end-on") or a mushroom (or loop) (two chain end attached-PEG or "side-on") conformation is presented as the highest steric protection against protein adsorption due to the steric hindrance of heavily hydrated chains of PEG molecules and their flexibility in terms of free rotation of individual polymer units around inter-unit linkages.⁹⁷

The polymer chain flexibility correlates with its ability to squeeze nonlinked water molecules out of the molecular dynamic "cloud" formed over the surface, making it impermeable for other solutes which require free water for diffusion such as the plasma proteins.⁹⁸ The structured water molecules, that are hydrogen bonded to the ether oxygens of PEG, are believed to form a protective hydration shell around the molecule creating an excluded volume.



FIGURE 6.9 Different types of PEG conformational clouds. The degree to which the coated surface resists protein adsorption depends on the molecular weight of PEG chains, their surface density and their conformation end-on (a) or side-on (b).

As the molecular weight of a PEG molecule rises, the cloud temperature, which is the temperature at which the polymer separates from the water solution, also increases, suggesting an increased amount of structured water and, therefore, different properties depending on PEG weight.⁸²

Independent of the size, computer simulations suggest that a relatively small number of molecules of a hydrophilic flexible polymer is sufficient to decrease the number of direct collisions of colloid surface with opsonizing proteins. When polymers are more rigid, like dextran for example, protective capabilities are much lower.⁹⁹ Indeed, the number of possible conformations for such polymers is lower and transitions from one conformation to another proceed at a slower rate than for a flexible polymer, which could allow the diffusion of plasma proteins between dextran molecules. In the same way, it was shown that branched PEGs are less effective at protein rejection than linear PEGs due to smaller exclusion volumes consistent with restriction in conformational freedom for the branched compounds.¹⁰⁰

6.3.2.1.2 Protein–Particle Interactions in the Presence of Poly(ethylene glycol)

Liposomes were the first carriers coated by PEG and on which the mechanism of PEG protective action was described. Phospholipids substituted with PEG chains of different molecular weights (1000–5000) and different percentages of the total lipid¹⁰¹ allow the obtention of circulating half-lives of up to 45 h in humans for PEGylated liposomes, as opposed to a few hours or even minutes for conventional liposomes. This longer plasma half-life, almost independent of the injected dose and of the particle diameter, comprised between 50 and 300 nm, allows the drug carrier to reach, eventually, tissues or organs other than MPS organs. This type of liposome can increase the circulating half-life of peptides and proteins, such as the cytokine interleukin-2¹⁰² or vasopressin which is a small peptide.¹⁰³

Many effects found for liposomes can be applied to nanoparticles. PEG can be introduced to their surface in two ways: (1) by adsorption or incorporation of surfactant polymers such as the poloxamer and poloxamine series (Figure 6.4) via hydrophobic interactions, (2) by the use of block or branched copolymers, usually based on polyesters, such as poly(lactic acid) (PLA). In both cases, hydrophilic fragments of adsorbed or coupled polymers are exposed into the solution providing steric protection, but possible desorption of PEG can occur in the first case.

An optimal protein-resistant effect of PEG on nanoparticle surfaces is considered to be dependent on both polymer chain length and surface density. It has been suggested that the lower surface density of high molecular weight PEG is more effective in reducing protein adsorption than the higher surface density of the low molecular weight polymers.¹⁰⁴ However, Jeon et al.^{96,105} suggest that the surface density has a greater effect than length on steric repulsion and Van der Waals attraction. The particle size and surface curvature can also affect the mobility and conformation of the PEG chains which show higher mobility on smaller particles due to more lateral movement.¹⁰⁶

Length and density of PEG chains of nanospheres prepared from PLA-PEG have been optimized to reduce their interactions with plasma proteins and to increase their circulating half-life.^{107,108} An optimal distance has been found between two terminally attached chains (2.2 nm), and any further increase in the chain length (over 5000 Da) did not confer any additional advantage at optimal surface density.¹⁰⁸ Similar calculations were made with nanocapsules (capsules of polymer containing an oily core) containing poly(lactide)poly(ethylene glycol) copolymers.¹⁰⁹ However, the degree of PEGylation of different copolymers used in PEG-coated poly(alkyl cyanoacrylate) nanoparticles did not to affect the *in vivo* behavior of the nanoparticles, whereas in vitro data had shown a modification of plasma protein adsorption depending on the density of PEG.^{110,111}

A lower level of complement activation was shown in vitro by nanoparticles presenting a mushroom conformation of PEG at their surface than for a brush conformation¹¹² despite the reduced mobility of the PEG chains discussed



FIGURE 6.10 Examples of relationships between protective polymer chain conformation, hydration, density, length, flexibility and resistance to protein adsorption. (a) Flexibility and high hydration of long chains allow the repulsion of the proteins. If flexibility is insufficient, structured water can be expulsed due to entropic instability and proteins can adsorb onto the surface. (b) A high density of adequate brush-like polymer chains constitutes a good hydrophilic sterical protection. The extreme opposite is the case where no protective chains are located at the surface of a hydrophobic particle. (c) The mushroom conformation can also constitute good protection if the flexibility and the coverage density are good. However, the presence of nucleophilic groups exposed at the surface will lead to high protein adsorption.

before (see also Section 6.4.2.2.1). In another in vitro study dealing with nanoparticles made of triblock PLA/PEG copolymers with short PEG chains at their surface, the postulated loop conformation also proved to be as resistant to cellular uptake as nanoparticles made of diblock copolymers with PEG chains in the brush conformation.¹¹³

Finally, relating conformation, hydration, density, length and flexibility of PEG chains to resistance to protein adsorption (Figure 6.10) is expected to lead to the elaboration of a model and to provide the knowledge necessary for the design of different protein-resistant coatings.

6.3.2.1.3 Fate of Polymer-Coated Long-Circulating Colloidal Drug Carriers

All polymer-coated long-circulating particles are at some point removed from the blood by macrophages of the MPS.⁶² One of the possible explanations is related to the polymer coating. When polymers are adsorbed, the particle coating may vary from one polymer sample to another, due to the statistically different strength of the noncovalent binding of individual macromolecules.

A possible displacement of these molecules by some plasma proteins is therefore possible, decreasing steric protection. When polymers make up of the particles or are covalently linked, swelling, degradation, erosion, or chemical breakdown may induce morphological and surface changes favorable for opsonization and phagocytosis.

The future of nonbiodegradable polymers used in nanoparticle surface engineering is ignored in most studies. The major assumption is that polymer excretion via the renal system takes place.⁶⁸ Acute and chronic pharmacological effects such as complement activation-related pseudoallergy (see also Section 6.4.3), modulation of gene activation, enzyme activity, and signal transduction are rarely studied. In addition, if macrophages are unable to digest the material they phagocytose, masses of macrophages collect in the tissue, forming a granuloma, in an attempt to wall off the material from the rest of the body.^{114,115} Examples of biocompatibility data concerning gene and DNA carriers can be consulted in the literature.^{116,117}

6.3.2.2 Biomimetic Systems

Besides PEG, other isolated polymers have been considered to decrease biorecognition. Albumin,¹¹⁸ semitelechelic poly(N-(2-hydroxypropryl) meth-acrylamide)s (ST-PHPMA),¹¹⁹ synthetic polymers of the vinyl series, such as polyacrylamide (PAA) and poly(vinyl pyrrolidone) (PVP),^{98,120} may serve as examples of potentially protective polymers.

Long circulating carriers can also be based on the principles of Nature. For example, healthy erythrocytes fulfill their function of transporting oxygen and are known to evade the macrophages of the immune system; they have a life span of 110 to 120 days, thanks to physicochemical and physiological factors including surface as well as bulk properties (composition, charge, shape, deformability, etc.).⁶² In 1978, Van Oss¹²¹ showed that many pathogenic bacteria possess a surface that consists of a highly hydrophilic hydrated layer of proteins, polysaccharides and glycoproteins which reduces their interaction with blood components and inhibits phagocytosis of the bacteria by the cells of the MPS. Nowadays, considerable information has become available from pathogenic microorganisms that employ various surface strategies to avoid recognition by macrophages as, for instance, several viruses which express complement regulatory proteins in order to reduce complement attack.¹²² As a result, another strategy for preparing long-circulating colloidal systems can be considered as biomimetic, in that it seeks to imitate cells or pathogens that avoid phagocytosis by reducing or inhibiting complement activation.

6.3.2.2.1 The Use of Different Natural Compounds

In the field of liposome research, prolonged systemic circulation in mice can be achieved by imitating the surface composition of the red blood cells via the incorporation of monosialoganglioside GM1 into the lipid phase.¹²³ Similarly,

intact or deacylated poly(sialic acid)s and shorter chain derivatives can be used to augment the half-lives of drugs, small peptides, proteins and liposomes in the blood circulation, thus prolonging their pharmacological action.¹²⁴ These systems may possess circulating half-lives that are as long as liposomes bearing PEG, A similar strategy has been applied to poly(isobutyl cyanoacrylate) nanoparticles where orosomucoid, a sialic acid-rich glycoprotein, was used. However, this approach was not successful because of desorption in the presence of plasma proteins.¹²⁵ Nanoparticles of PLA and poly(L-lysine)-grafted-polysaccharide were also developed for DNA delivery.¹²⁶ The polynucleotide adsorption capacity of the nanoparticles was increased with increasing polysaccharide contents, and nanoparticles exhibited resistance against self-aggregation and nonspecific adsorption of serum proteins, presumably due to the polymer brush effect and/or the exclusion effect from the polysaccharide graft chains.

Another biomimetic approach is the use of heparin known, as previously shown, as an inhibitor of several steps of the complement cascade. Amphiphilic copolymers were synthesized by polymerization of heparin and methyl methacrylate monomers.¹²⁷ In a defined range of concentrations, the copolymers formed submicronic particles which where stable in the absence of added surfactant. As expected, heparin graft nanoparticles produced a nonactivating surface (see Section 6.4.2.2.2).¹²⁸ They avoided both adsorption and internalization processes by macrophages in vitro,¹²⁹ and were long-circulating *in vivo* (half-life of 5 h or more compared with a few minutes for control poly(methyl methacrylate) particles).¹³⁰

6.3.2.2.2 The Use of Dextran

Similar amphiphilic copolymers were synthesized by polymerization of dextran and methyl methacrylate monomers.¹²⁷ While they were complement activators,¹²⁸ they showed long-circulating properties probably due to a steric hindrance effect caused by a brush-like or loop-like arrangement of flexible end-attached polysaccharide chains, similar to those obtained with PEG, No physico-chemical studies were carried out in order to define the real conformation of dextran at the surface of these particles.

A similar prolongation of circulation times has already been observed in early studies with dextran-conjugated liposomes; the density of the steric brushes of dextran on the surface being a determining factor.³¹ Another example of prolonged circulation in the blood thanks to the hydrated brush-like structure of dextran molecules, is the successful development of superparamagnetic iron oxide particles (or ferrofluids) that are used in magnetic resonance imaging (Figure 6.6).⁸⁰ Their small size (iron core of 5 nm and an unimodal hydrodynamic radius of 25 nm) and hydrophilic nature confer them blood half-lives of 3 to 4 h.

The end-on and side-on configurations of dextran bound to plane surfaces have been compared ¹³¹ but not at the particle surface. As in the case of PEG, relationships between conformation, density, molecular weight, flexibility and

protein rejection properties (Figure 6.9 and Figure 6.10) have to be investigated when the use of dextran at the surface of colloidal drug carriers is expected to design long-circulating particles.

6.3.2.3 Targeting Systems

Currently, the principal schemes of drug targeting include the direct application of a drug into the affected zone, passive drug targeting (spontaneous drug accumulation in the areas with leaky vasculature, or enhanced permeability and retention (EPR) effect), physical targeting (based on abnormal pH value and/or temperature in the pathological zone), and magnetic targeting (or targeting of a drug immobilized in paramagnetic materials under the action of an external magnetic field). A challenge in the development of novel colloidal carrier systems is now to design targetable carriers in which pilot molecules are installed on their surface to achieve a specific-binding property to target cells (Figure 6.11). Ligands, such as monoclonal antibodies, sugars and lectins, or growth factors, can be coupled to these colloidal systems via some endfunctionalized PEG, allowing the conjugation of the pilot molecules at the tethered end of the hydrophilic segments. Promising results have been obtained in vitro although these have often not been confirmed in vivo for the moment. For example, PEG-liposomes bearing folic acid for which receptors are overexpressed in a number of human tumors, have been shown to be effective carriers for intracellular delivery of nucleic acids and anti-cancer drugs to tumor cells in vitro.132

A solution to avoid MPS capture of these targeting systems might be to limit the degree of substitution so as to avoid recreating a surface on which opsonization can occur¹³³ and to use longer PEG chains to carry the ligand than the PEG chains used to confer long-circulating properties (Figure 6.11).¹³⁴



FIGURE 6.11 Schematic representation of a targetable carrier on which pilot molecules are installed in order to achieve a specific-binding property to the target cells. The ligands usually employed are monoclonal antibodies, sugars, lectins, or growth factors. PEG chains can be used to confer long-circulating properties to the carrier and also to make a covalent bond with the ligand.

An example of a cell-surface receptor which can be targeted by immunoliposomes is the her2 (ErbB2) antigen.¹³⁵ A phospholipid bearing a PEG chain terminated by an anti-her2 antibody fragment was inserted into preformed commercially available stealth doxorubicin-loaded liposomes (Doxil[®]) which led to dramatically increased concentrations of the drug in her2-overexpressing tumor cells. However, coupling a large protein to the carrier surface can have drawbacks such as the weaker diffusion of particles within the tumors, rapid clearance from the circulation and immunological and pseudoallergic reactions.¹³⁶ Most of these drawbacks can be avoided by a two-step procedure using bispecific antibodies, also called Affinity Enhancement System (AES),¹³⁷ which combines two immunological recognition functions in the same system. The bispecific antibodies are firstly injected and fix themselves to tumor antigens. The colloidal carrier carrying a hapten on its surface is injected in a second time and is expected to recognize specifically the antigen–antibody complexes by the way of the second fragment of the antibodies.

6.4 INTERACTION OF COLLOIDAL DRUG CARRIERS WITH THE COMPLEMENT SYSTEM

Complement C3 and immunoglobulin G are found to be the major proteins which adsorb onto the surface of colloidal drug carriers, along with fibrinogen, albumin, and apolipoproteins after incubation in human plasma and serum.¹³⁸ On exposure to blood, particles of differing surface characteristics, size and morphology will attract different arrays of these opsonins as well as other plasma proteins. Opsonic activity is one of the most important functions of the complement system besides its other activities including the initiation of membrane damage, the induction of inflammation and the regulation of adaptive immunity. As a result, it is of interest to determine whether interactions between complement and colloidal drug carriers can influence the design of long-circulating carriers.

6.4.1 AN EXAMPLE OF COMPLEMENT ACTIVATION Assay: The CH50 Test

Activation of the complement sequence leads to the consumption of complement components which, in turn, can lead to a decrease in their concentration. Thus, the determination of this consumption can indicate whether the complement system has been activated by a pathogenic mechanism or, as in our case, by colloidal drug carriers.

To be useful in diagnostic work, screening procedures for the detection of complement deficiencies should be simple and rapid, and should clearly distinguish between defects within the functional units of the complement system, i.e., the classical activation pathway (C1, C2, C4), the alternative activation pathway (C3, factor B, factor D, properdin), and the terminal sequence (C5–C9). Hemolytic assay systems are commonly used for this purpose. Simplified procedures suitable for relatively large scale investigations

of patients have been described¹³⁹ and can be adapted for testing the biocompatibility of colloidal drug carriers exposed to blood for therapeutic purposes.¹⁴⁰

Hemolytic dosage CH50 (complement hemolytic 50%) can be carried out in most laboratories without special equipment. It allows the measurement of the functional activity of the global classical complement pathway components. The principle of the procedure is based on the fact that, when sheep erythrocytes are sensitized with an antibody against sheep erythrocytes, an antigen-antibody complex is formed. This complex, when exposed to the complement in human serum, will activate the classical pathway, resulting in the lysis of erythrocytes and the release of hemoglobin. The CH50 units represent the concentration by ml of serum of hemolytic complement units able to cause 50% hemolysis of a fixed volume of these sheep red cells. If serum is previously put in contact with activating particles, their opsonization occurs, so therefore less complement proteins remain in the serum to lyse the sheep red cells, and the CH50 units decrease. After determining the CH50 units remaining in the serum, the results can be expressed in terms of the consumption of CH50 units. In order to compare particles with different mean diameters, this consumption can be expressed in terms of the surface area of the particles.¹⁴⁰ An example of CH50 unit consumption by poly(methyl methacrylate) nanoparticles of two different sizes (50 and 100 nm) (Bangs Laboratories, Inc.) in function of surface area is given in Figure 6.12 (data not published).



FIGURE 6.12 Consumption of CH50 units in the presence of poly(methyl methacrylate) nanoparticles of two different sizes (50 and 100 nm) as a function of surface area. Increasing amounts of nanoparticles were incubated for 1 h at 37° C with human serum diluted 1:4 in veronal buffered saline. For the same surface of 1200 cm^2 , 100% consumption occured for 100 nm nanoparticles while only 30% consumption was attained by 50 nm ones. This difference can be related to the curvature of the particles that plays an important role in the efficiency of C3 convertases to bind to the surface.

It is important to notice that materials able to inhibit sheep red cell lysis like heparin¹²⁸ as well as materials able to lyse them cannot be studied by the CH50 test.¹⁴¹ Except in these cases, differences between weak and strong colloidal activators due to different compositions, types of coating, polymer conformation, particle curvatures, and other physico-chemical variations can be accurately assessed by using this technique.

In addition to hemolytic assays which are total functional dosages, the ELISA procedure appears to be a simple, rapid, and reliable immunochemical dosage for the detection of particular component deficiencies.¹⁴² Radioimmune assays (RIA), immunoblotting and bioassays can also be used for complement component assays.¹⁴³

6.4.2 INTERACTION WITH COLLOIDAL DRUG CARRIERS

In nature, "foreign" substances are almost always presented as particles, ranging in size from viruses and bacteria to fungal spores, protozoa or tumor cells. The best complement activators are themselves in the form of particles even if soluble activators are known (Table 6.3). The examples of colloidal drug carriers discussed thereafter are summarized in Table 6.4 and Table 6.5.

Factors	Strong activation	Weak activation	References
Charge	Negative/positive	Neutral	Volanakis et al. 1979 Chonn et al. 1991 Bradley et al. 1998
Cholesterol	Presence	Absence	Mold et al. 1981 Kovacsovics et al. 1985 Wassef et al. 1989 Chonn et al. 1991
Saturation of acyl chains	Unsaturated (in human)	Saturated Unsaturated (in rat)	Chonn et al. 1991 Devine et al. 1994
Size	Large	Small	Senior et al. 1987 Harashima et al. 1994 Marjan et al. 1994 Harashima et al. 1996
Number of injections	Repeated injections	One injection	Dams et al. 2000 Laverman et al. 2001 Price et al. 2001 Ishida et al. 2003

The role of different factors in the activation of the complement by liposomes

TABLE 6.4

TABLE 6.5 Different strategies used to decrease the complement activation by nanoparticles

Authors	Carrier composition (core/coating)	Method	Strategy
Vittaz et al. 1996	PLA-PEG	CH50	High density brush-like configuration
Gbadamosi et al. 2002	Polystyrene-PEG	CH50	High density brush-like configuration
Peracchia et al. 1997	Poly(iBCA*)-PEG	CH50	Mushroom configuration
Ameller et al. 2003	Polyesters-PEG	SDS-PAGE	Decreasing hydrophobicity
Mosqueira et al. 2001	Oily core PLA-PEG	Crossed immuno- electrophoresis**	Brush-like configuration Role of lecithin
Passirani et al. 1998	PMMA-Dextran	CH50 and crossed immuno- electrophoresis	High density brush-like or mushroom-like configuration
	PMMA-Heparin		Idem + inhibitory effect
Chauvierre et al. 2003	Poly(iBCA)- Dextran Poly(iBCA)-	Crossed immuno- electrophoresis	Idem Passirani et al.
	nepann		

*Isobutyl cyanoacrylate.

**After incubation of nanoparticles with serum diluted 1: 4 in veronal buffered saline, crossed immuno-electrophoresis is assessed on 1% agarose gel. The second-dimension electrophoresis is carried out in the presence of a polyclonal antibody to human C3, recognizing both C3 and C3b. The relative sizes of the immunoprecipitation peaks revealed by Coomassie blue staining give a semi-quantitative estimation of the proportions of native and cleaved C3 (C3b).

6.4.2.1 Liposomes

The activation of the complement system by liposomes has been reported in human, rat, and guinea pig serum/plasma.¹⁰ Important factors in the activation of the complement cascade have been highlighted as a negative charge, the presence of cholesterol, saturated acyl chains, acyl chain length and the size of liposomes (Table 6.4). Besides the important role of complement of enhancing uptake by MPS cells, the assembly of the membrane attack complex can produce a lytic pore which, in turn, induces the lysis of liposomes or the release of liposomal contents.

Both negatively and positively charged liposomes activate the complement system, whereas little activation is usually detectable with small, neutral ones.^{23,144,145} Liposomal triggering of the classical pathway with or without active participation of antibodies has been described in numerous studies,^{21–23} including one reporting that naturally-occurring cholesterol antibodies were responsible for liposome-induced complement activation in pigs.¹⁴⁶

Unsaturated lipids are more potent complement activators than saturated ones in human serum,²³ but less potent in rat serum.¹⁴⁷ Indeed, the role of complement has been demonstrated for several types of liposomes but a species difference in the hepatic uptake mechanism exists, especially between rats and mice. The smaller the animal size, the higher the hepatic uptake clearance per unit of body weight by mechanisms which can be opsonin-dependent as well as opsonin-independent.¹⁴⁸

Vesicle size is one of the major factors in determining the distribution of liposomes in the body. In general, larger liposomes are eliminated from the blood circulation more rapidly than smaller ones.¹⁴⁹ It was shown that the enhanced uptake of larger liposomes by the liver is governed by the extent of opsonization which depends, in turn, on the size of liposomes. Size-dependent opsonization via activation of the complement system was found in both the classical and alternative pathways.¹⁵⁰ A size-dependent difference in the number of recognition sites could exist due to a different curvature in the local area on the surface of liposomes. Because complement activation is the result of a chain reaction involving several complement components, enough local room is required (for instance, dimensions of the convertase C3bBb is about 14×8 nm) (Figure 6.13).^{151,152}



FIGURE 6.13 Schematic representation of C3bBb convertase (about 14×8 nm) in comparison to 20, 50, and 100 nm particles. In addition to the potential repulsion exerted by a protective polymer, the size and the curvature of the particle play an important role. A size-dependent difference in the number of recognition sites for complement proteins on the surface of the particle could exist due to a different curvature. Moreover, complement activation is the result of a chain reaction involving several complement components that need sufficient room to bind to the particles. All these reasons can explain why the smallest particles are generally the stealthiest.

It was shown recently that the intravenous administration of sterically stabilized PEG liposomes significantly altered the pharmacokinetic behavior of subsequently injected PEG liposomes in a time- and frequency-dependent manner.¹⁵³ This accelerated clearance of PEGylated-liposomes on repeated injection was also observed after transfusion of serum from rats that had received PEGylated-liposomes one week earlier.¹⁵³ This may be a general characteristic of liposomes, unrelated to the presence of PEG^{154,155} It is likely that cellular immunity (Kupffer cells) and humoral immunity are required to cause this phenomenon.^{156,157} Therefore, these findings may have a considerable impact on the clinical application of liposomal formulations that are administered repeatedly.

6.4.2.2 Nanoparticles

It is worth noticing that the circulating half-lives that can be achieved with nanoparticulate systems are, for the moment, not as long as those obtained with liposomes.⁶⁰ Could this be due to the fact that the more rigid surface of the polymeric systems is a more powerful complement activator?

Little is known about the exact mechanisms of complement activation of most of the materials used in colloidal drug carriers. At least complement activation has been evaluated in the following examples (Table 6.5).

6.4.2.2.1 PEGylated Polymeric Particles

As previously discussed, PEGylation of systems greatly improves the stability, efficacy, and efficiency of various systems and makes them suitable for delivery of bioactive species in the body for much longer periods, with reduced immunogenicity, antigenicity, and toxicity in the body. Moreover, due to the transient, flexible, and rapidly changing structure of PEG, the immune system would have difficulties in modeling an antibody around it.¹⁵⁸

By using complement hemolytic assays, Vittaz et al.¹⁴⁰ showed that native particles made from the diblock copolymer PLA-PEG which escape from fast phagocytosis, also consume a very low amount of complement, provided that the average PEG surface density is sufficient. On the other hand, a fast and strong complement consumption occurred in the presence of PLA particles stabilized by a simple surfactant absorbed on the surface (Pluronic[®] F68) probably following a fast desorption/exchange of the surfactant by some complement proteins.

The different possible PEG conformations on the colloidal drug carrier surface are presented in Figure 6.9 and Figure 6.10. Their role in complement activation has been specifically studied by a few authors. It was shown that, in a heterogeneous population of PEGylated microspheres that differed in surface characteristics, populations bearing a predominant surface of PEG molecules as high-density mushroom-brush intermediate and/or brush configuration activated the complement system poorly, whereas in a predominant mushroom configuration, PEG surfaces were potent activators and were prone to phagocytosis.¹⁵⁹ The consumption of complement by PEG coupled to poly(isobutyl cyanoacrylate) nanoparticles, with PEG in end-on and side-on conformation was also evaluated.¹¹² Surprisingly, the PEG side-on configuration showed lower complement consumption, suggesting an insufficient end-on conformation. Density could be implicated but also the composition of the particle core since, in both conformations, PEG protection was much less effective than with PLA particles used in Ref. 140. Actually, different polyester cores of PEGylated nanoparticles studied by a few authors showed an inhibition of complement protein adsorption related to the different levels of core hydrophobicity.¹⁶⁰

Oily core nanocapsules made of PLA or PEG-PLA were also studied for complement consumption and were shown to have protein-rejecting properties superior to those of nanospheres for an equivalent surface area. The testing of longer PEG chains (20 kDa) showed them to be more effective, even at lower surface density, in suppressing complement activation, thanks to a predominant brush-like PEG configuration which may sterically suppress the deposition of large C3 convertases. A role in reducing C3 activation has also been suggested for lecithin constituting the core of the nanocapsules.¹⁶¹ This could be correlated to other results showing improved properties for the prolongation of circulation and a smaller amount of serum protein adsorbed for lecithin-coated polystyrene nanoparticles in contrast to uncovered polystyrene ones. Once more, differences of hydrophobicity play an important role here.¹⁶²

6.4.2.2.2 Biomimetic Particles

Although some studies demonstrated that not only OH density but also other features linked to the type of polymeric backbone are involved in complement activation by OH-bearing surfaces,¹⁶³ complement activation of both the alternative and classical pathways is mainly thought to be a function of the availability of hydroxyl groups present on a polymeric surface.¹⁶⁴ The majority of biomimetic surface coating polymers used to date (mainly polysaccharides as described in Section 6.3.2.2) are rich in hydroxyl groups and their complement activation therefore has to be taken into account.

Soluble dextran and heparin are known to have respectively activating and inhibiting properties towards complement (Section 6.2.4). They were covalently bound to polymeric poly(methyl methacrylate) nanoparticles in order to examine if their properties could be maintained.¹²⁸ Concerning heparin, it was concluded that it retained its inhibiting properties towards complement after binding to nanoparticles and that they were even increased by an unknown mechanism, perhaps as a result of cooperative effects between chains bound side by side on the same particle. Second, dextran-graft nanoparticles were compared to soluble dextran and to crosslinked dextran (Sephadex[®]) and were shown to be weak activators of complement, like soluble dextran. The great difference with Sephadex[®] (known as a strong activator) was attributed to two different physico-chemical characteristics: size and conformation. Indeed,

Sephadex[®] beads were around 30 um and dextran-nanoparticles around 80 nm. As already shown in Figure 6.12 and Figure 6.13, size is an important factor in complement activation. The radius of curvature of nanoparticles could limit the formation or efficiency of amplifying C3 convertase sites and/or recognition of the surface by the antidextran antibodies that have been shown to modulate complement activation on Sephadex[®].^{37,38} Otherwise, the cross-linked dextran chains of Sephadex® have little flexibility and also have good lateral accessibility of the outermost OH chains to proteins. The dextrannanoparticles are composed of self-organized copolymers carrying the dextran chains on the surface as a dense brush-like or mushroom-like layer. Such configurations would keep a higher flexibility of the chains when compared to the cross-linked chains and thus reduce their accessibility to the reactive C3 fragments. Heparin-graft nanoparticles were probably in the same configuration and their increased long-circulating properties, in comparison with dextran-graft nanoparticles, was attributed to their inhibitory effect.¹³⁰ Recently, heparin and dextran were tested at the surface of poly(isobutyl cyanoacrylate) nanoparticles, which led to the same conclusions concerning complement activation.¹⁶⁵

6.4.3 CONSEQUENCES OF COMPLEMENT ACTIVATION

Considering that complement activation is a major pathogenic factor in many cardiovascular and hematological variations and in the respiratory distress syndrome that often develops in trauma and shock, particle-induced complement activation may be a critical safety issue. Although a weak complement fixation may not necessarily lead to particle clearance from the blood, complement activation is associated with the release of anaphylatoxins which are thought to be responsible for the observed pseudoallergic reaction following intravenous injection of long-circulating vesicles as marketed liposomal formulations.⁶⁸ Numerous drug-induced immediate hypersensitivity reactions (HSR) are non-IgE-mediated and arise at first exposure to the drug without prior sensitization, hence the denomination of pseudoallergic reactions. They can be caused by certain liposomal formulations of intravenous drug and imaging agents, micelle-forming amphiphilic lipids or block-polymer emulsifiers and iodinated radiocontrast media.¹³⁶ All these systems have the capacity to activate the complement and the reactions they cause can be also called "complement activation-related pseudoallergy" (CARPA). Recent studies on liposome-encapsulated hemoglobin (potential blood substitute). which are subject to CARPA, provided a model for the biocompatibility testing of pharmaceutical products that may cause unexplained hypersensitivity reactions.¹⁶⁶ It is therefore of great interest to develop coating systems that do not activate complement.

However, it is important to notice that, in addition to significant interspecies variations, great variations among individuals exist. Variation in complement activation in the sera of healthy individuals by various poloxamers and poloxamines has been demonstrated.⁶² Impurities in copolymers or

derivatives of the copolymers generated in the manufacturing process have been proposed to be responsible for activation of the complement system.¹⁶⁷ Interestingly, removal of some impurities by dialysis or organic extraction resulted in further activation of the complement system in some individuals. These reactions could be related to a hypersensitivity to poloxamer/ poloxamine-based nanoparticles.

6.5 CONCLUSION

Even if a long circulation time is generally correlated to weak complement activation, various studies have indicated an association of opsonic molecules, such as components of the complement system and immunoglobulins, with stealth systems.¹⁵⁷ For instance, Doxil[®], a PEGylated liposome formulation with encapsulated doxorubicin which has a biphasic circulation half-life of 84 min and 46 h, is actually a strong activator of the human complement system with activation taking place within minutes.¹⁶⁸ In the few examples cited before, it also appears that PEGylation cannot totally prevent complement activation^{112,140,161} and that the hydrated polymeric "molecular cloud" is not completely inert (Van der Waals and hydrophobic interactions, hydrogen bonds, etc.).⁶⁸ Despite these facts, certain particles can circulate for several hours, as much as 45 h in humans.⁶² So, how can these opsonized systems circulate for long periods of time?

First, in some cases, C3 can be detected on the surface of a particle by different technical tests, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or immunoblotting but it may have bound in a noncovalent manner, a process that does not necessarily lead to complement activation. Second, when C3 is bound in a covalent manner, competition between the surface-bound and free C3b for phagocytic complement receptors is possible.⁶⁸ as well as degradation of surface-bound C3 to fragments inhibiting recognition.¹⁶⁹ In addition, complement fixation on PEG-bearing particles may occur in a cryptic location inaccessible for ligation to complement receptors such as in the complement fixation by Staphylococcus aureus.¹⁷⁰ The limited concentration of the blood opsonin and favorable interactions between surface-bound polymers and blood dysopsonins also contribute to the prolonged circulation time of sterically protected particles. Finally, C3b may interact with CR1 expressed on human erythrocytes, which could explain their prolonged residence in the systemic circulation system.¹⁷¹ Concerning erythrocytes, the fact that they are not phagocytosed, even by activated Kupffer cells, could rather hold the key for the design of a truly synthetic long-circulating colloid and, at the same time, end the long search for artificial blood.

One additional possible reason why a certain percentage of the engineered particles is prone to phagocytosis can be related to an inadequate surface coverage by protecting molecules or to the displacement, erosion or chemical degradation of this coating. Future efforts to yield more homogeneous particles with regard to their surface properties are required, especially for colloidal drug delivery systems containing peptides, proteins, oligonucleotides or genes, which often influence the surface of the carrier.

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7 Biological Membranes and Barriers

Yadollah Omidi and Mark Gumbleton

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7.1 INTRODUCTION

The successful delivery of biologics (polypeptides or polynucleotides) as therapeutic entities requires the delivery system or technology to overcome the biological barriers that such agents will encounter when they are administered in vivo. There are currently many good examples of biologics that have reached the market and are available for prescribing, including peptides, recombinant blood factors, enzymes, hormones, cytokines, vaccines, and antibody-based products. Inevitably future advances in the delivery of biologics will require more specific exploitation of the body's biology for effective patient therapy. The systemic administration of nonviral gene vectors represents a particular problem that may not be overcome by simply over-engineering the delivery system. The future research into nanomedicines will require integration between physicochemistry, biology and engineering, and is an exciting prospect for all pharmaceutical scientists involved in this area. Each must equip themselves with at least the basics of the contributing disciplines. This chapter served only as a brief outline of the biological nature of membranes and barriers than biologic therapeutic agents.

7.2 COMPOSITION AND ARCHITECTURE OF CELL MEMBRANES

7.2.1 LIPID BILAYER

The structure and morphology of a cell membrane has been defined by the "fluid mosaic" model^{1,2} (Figure 7.1) which has served as a platform for the emergence of concepts in cell membrane lipid–lipid and lipid–protein interactions. Techniques such as x-ray diffraction, and transmission and freeze-fracture electron microscopy have reaffirmed the validity of the membrane bilayer model. Lipids spontaneously form into bilayers stabilized by hydrophobic forces driving the minimization in the surface area of contact between molecules of water and the hydrophobic acyl chains of lipids. The major types of lipid in cell membranes are phospholipids, cholesterol, and glycolipids; all are amphipathic with the "fluidity" (viscosity) of the lipid bilayer depending upon the nature and composition of its lipid components.



FIGURE 7.1 Schematic structure of lipid bilayer and the interactions of membrane proteins. Key: a, a protein bound noncovalently to anchor protein; b, a protein bound electrostatically to the bilayer; c, a protein bound hydrophobically to the bilayer; d, membrane protein membrane anchored by a short terminal segment; e, transmembrane protein comprising a single membrane-spanning domain; f, transmembrane protein comprising a multiple membrane-spanning domains; g, membrane anchored by covalently through a lipid chain.

A phospholipid molecule (e.g., phosphatidylcholine), possesses a polar head group (e.g., choline-phosphate-glycerol), and two hydrophobic fatty-acid tails or chains. The shorter the hydrophobic tails and the higher degree of unsaturated *cis*-double bonds in the fatty-acid chain then the lesser tendency for hydrophobic chains of adjacent phospholipids to interact and the lesser tendency for them to pack together, i.e., the more "fluid" is the membrane. The plasma membrane of most eukaryotic cells contains a variety of phospholipids, for example those based on glycerol include phosphatidylcholine, phosphatidylserine (possesses a net negative charge at physiological pH) and phosphatidylethanolamine. Sphingomyelin is a membrane phospholipid based upon ceramide. Cholesterol is a major component of plasma membranes (15–20% of total lipid by weight) and a key determinant of membrane "fluidity."

The lipid composition of cell membranes is asymmetric with respect to the inner (cytoplasmic) and the outer (exofacial) leaflets. This asymmetry in phospholipid composition generated during plasma membrane synthesis within the endoplasmic reticulum. Almost all the lipids possessing a choline headgroup (phosphatidylcholine and sphingomyelin) are in the exofacial leaflet, whilst almost all of the phospholipids that contain a terminal primary amino group (phosphatidylethanolamine and phosphatidylserine) are in the cytoplasmic leaflet. This asymmetry extends to the properties associated with the phospholipid acyl chains, i.e., phosphatidylethanolamine and phosphatidylserine display a higher level of unsaturation. Further, since phosphatidylserine is the only lipid displaying a net negative charge then the inner leaflet is more negatively charged than the outer leaflet. This asymmetry appears critical for a number of signal transduction functions of the cell.

Glycolipids are oligosaccharide-containing lipid molecules which are located exclusively in the outer leaflet of the membrane bilayer, i.e., the leaflet exposed to interstitial fluid that bathes all cells, with the polar sugar groups exposed at the surface. In animal cells almost all glycolipids are based upon ceramide (e.g., sphingomyelin). These glycosphingolipids have a general structure comprising a polar head group and two hydrophobic fatty-acid chains. Glycolipids are distinguished from one another by their polar head group which consists of one or more sugar residues. The most widely distributed glycolipids in the plasma membranes of eukaryotes are the neutral glycolipids whose polar head groups consist of 1 to 15 or more uncharged sugar residues.

7.2.2 LIPID RAFTS

Lipid rafts are specialized membrane domains enriched in certain lipids, cholesterol, and proteins. The existence of lipid rafts was first hypothesized in 1988 by Simons and associates,^{3,4} but caveolae—a specialized form of lipid raft were first observed in the 1950s.^{5,6} The fatty-acid chains of lipids within the rafts are more extended and pack together more tightly, creating domains with higher order and forming a gel phase in which there is very little lateral movement or diffusion. Sphingolipids, and other lipids with long, straight acyl chains are preferentially incorporated into the rafts. The association of cholesterol within this gel phase confers further microdomain properties. Rafts therefore are considered as specialized lipid islands or membrane domains existing as a separate ordered phase that floats in a sea of poorly ordered lipids. Many functions have been attributed to rafts, including that of vesicular transport and regulation of signal transduction pathways.^{7,8}

Caveolae are lipid rafts enriched with cholesterol, glycosphingolipid, and importantly the protein caveolin (which is a key structural and functional element for caveolae). Using a variety of techniques, including those exploiting the detergent resistant nature of the lipid rafts, numerous other proteins involved in cell signal regulation have been localized to caveolae membrane domains. Caveolae have also been shown to be sites not only regulating a number of signal transduction pathways but also in mediating the transport of ligands such as folate, cholesterol and in continuous microvascular endothelial cells, albumin.

The dynamic formation of liquid-ordered phase domains within the liquiddisordered matrix has been considered as an important regulator of molecular interactions in membrane trafficking. Cholesterol–sphingolipid rafts while probably not the only such domain, provide one of the most defined systems to study lipid-mediated protein trafficking. However little is known in terms of composition of individual proteins and lipids within a given raft "subclass," the residency time of a particular raft constituent within the domain, and also the mechanisms of crosstalk between bilayer leaflets. Cell membrane solubility properties, using different detergents, suggest that membrane domains of different solubility contain subsets of membrane rafts, which differ in their molecular compositions. It presently seems that there are at least three types of raft, i.e., caveolae, noncaveolae glycosphingolipid enriched membranes, and polyphosphoinositol rich rafts.

7.2.3 PROTEIN INTERACTIONS WITHIN THE LIPID BILAYER

Most of the specific functions of biological membranes are carried out by proteins. The amounts and types of protein in a membrane are highly variable. The density of a cell membrane is directly proportional to the amount of protein in the membrane, i.e., the higher the protein composition the greater the density. Membrane proteins associate with a lipid bilayer in many different ways (Figure 7.1).⁹ Transmembrane domain (TMD) proteins extend across the bilayer as a single α helix or multiple α helices. These TMD proteins comprise a major group of proteins divided into TMD super-families the members of which perform a wide range of functions, and would include, among others, G-protein coupled receptors, growth factor receptors, cell adhesions proteins, ion channels, transporters, and metabolic enzymes. For example, the xenobiotic efflux transporter, P-glycoprotein (P-gp) consists of twelve transmembrane spanning domains and two adenosine 5'-triphosphate (ATP) binding domains. The homology of the latter leading to the classification of P-gp as a member of the ATP-binding cassette (ABC) superfamily of transporter proteins.

The great majority of transmembrane proteins are glycosylated with the oligosaccharide chains exposed to the extracellular environment; P-gp is N-glycosylated which probably has important implications for membrane targeting, insertion and stability of this protein. Some integral membrane proteins are attached to the bilayer only by means of a fatty-acid chain, while others are attached covalently via a specific oligosaccharide. Integral membrane proteins can be released only by disrupting the bilayer with detergents or organic solvents.

All eukaryotic cells have carbohydrate on their surface membranes both as oligosaccharide chains covalently bound to proteins (glycoproteins) and as oligosaccharide chains covalently bound to lipids (glycolipids). The term glycocalyx is used to describe the carbohydrate–rich peripheral zone on the externally orientated surface of cell membranes. The glycocalyx is characterized by a net negative charge which results from the presence of sialic acid or sulfate groups at the nonreducing termini of the glycosylated molecules. This "blanket" of negative charge serves to protect the underlying membranes and has clear implications with respect to xenobiotic–membrane interactions as well as cell–cell communication.

7.3 GENERAL NATURE OF EPITHELIAL AND ENDOTHELIAL BARRIERS

7.3.1 EPITHELIAL BARRIERS

Biological barriers to drugs would include, amongst others: intracellular membrane barriers preventing drug access to certain subcellular compartments, e.g., nuclear membrane restricting access to the nucleus or endosomal membrane preventing access of endocytosed material to the cytoplasm; the plasma membrane limiting access of drugs to intracellular targets; metabolic degradation limiting the available fraction reaching the blood or lymph circulations, and ultimately the target tissue and cell. Aspects of these will be discussed throughout this article, but here we focus upon an overview of epithelial and endothelial barriers which respectively limit access of drug into the body and drug movement from blood into tissue.

The properties of the body's epithelial barriers vary greatly but may be classified morphologically as: (i) simple squamous epithelium comprising a single layer of thin flattened epithelial cells resting directly on the basement membrane, e.g., alveolar epithelium within which the type I cell possess an apical surface area of approximately $5000 \,\mu\text{m}^2$ and in the peripheral regions of the cell can be as thin as $0.25 \,\mu\text{m}$; (ii) simple columnar or cuboidal epithelium comprising a single layer of column-shaped or cuboidal-shaped epithelial cells resting directly on the basement membrane, e.g., columnar enterocytes of the intestinal tract which possess an apical surface area of $\sim 100 \,\mu m^2$ (excluding microvilli) and have a depth of approximately 25 µm. Exocrine glands are usually lined with simple cuboidal epithelium; (iii) stratified epithelium is common in areas of the body that need to withstand wear and tear. The epithelia are composed of several layers of cells with the top layers usually very flattened and it may or may not be keratinized (i.e., containing a tough, resistant protein called keratin). Only the basal cells of the epithelium are in contact with the basement membrane. The mammalian skin is an example of dry, keratinized, stratified epithelium. The lining of the mouth cavity is an example of an unkeratinized, stratified epithelium; (iv) pseudostratified epithelium which is an intermediate between simple and stratified epithelia. In pseudostratified epithelia there appears to be many layers of cells but actually all cells rest on the basement membrane though not all cells traverse the full depth of the epithelium. An example of pseudostratified epithelium is that within the conducting airways of the lung comprising cuboidal or columnar epithelial cells interspersed with mucus secreting goblet cells.

7.3.2 ANCHORING JUNCTIONS

The barrier properties of epithelial or endothelial cell membranes are only effective if adjacent cells within the epithelium or endothelium form intercellular junctions and cellular–extracellular matrix junctions. The anchoring junctions include: (i) desmosomes which are intercellular anchoring junctions linking two cells through cell adhesion proteins termed cadherins which extend from the cytoplasmic leaflet of one cell through to the cytoplasmic leaflet of an adjacent cell. The cadherins are linked intracellularly to intermediate cytoskeletal filaments composed of keratin or desmin that are themselves attached to membrane-associated attachment proteins that form a dense plaque on the cell membrane cytoplasmic leaflet; (ii) hemidesmosomes which link cell cytoskeleton to extracellular matrix. In electron micrographs they bear strong resemblance to half of a desmosome (hence the name), but are indeed quite distinct. Like desmosomes, they are linked to intermediate filaments in the cytoplasm, but in contrast to desmosomes, their transmembrane anchors are integrins rather than cadherins; (iii) Adherens junctions share the characteristic of anchoring cells through their cytoplasmic actin filaments. Similarly to desmosomes and hemidesmosomes, their transmembrane anchors are composed of cadherins in those that anchor to other cells and integrins in those that anchor to extracellular matrix. The intercellular adherens junction, also termed zonula adherens comprises a band of cadherin protein encirculating the cell and linking to intracellular actin filaments just below the plasma membrane. The linkage brought about through the zonula adherens is essential for functionality of the zonula occludins or tight junctional complexes that form above the zonula adherens more proximal to the apical cell surface. Adherens junctions that link cell to extracellular matrix display a spot-like morphology and are termed adhesion plaques. Through linkage to the cytoskeletal actin filaments, the adherens junctions are thought to participate in folding and bending of epithelial cell sheets.

In addition to anchoring junctions, cells also possess gap junctions for intercellular communication. Gap junctions are arrays of channels that permit small molecules to shuttle from one cell to another and thus directly link the interior of adjacent cells. Gap junctions allow electrical and metabolic coupling among cells as signals initiated in one cell can readily propagate to neighboring cells. In general, the upper limit for passage through gap junctions is roughly 1000 daltons (Da). Aside from ions, important examples of molecules that readily pass include cyclic AMP (329 Da), glucose-6-phosphate (259 Da) and nucleotides (250-300 Da). The bystander effect as exploited in cancer gene therapy protocols, e.g., transfection with herpes simplex virus-thymidine kinase which converts gancyclovir into a toxic metabolite, relies for efficacy on the intercellular transfer of the generated toxic metabolite via gap junctions. The major protein in gap junctions is connexin. All connexins share a common structure of having four membrane-spanning domains. A connexon is formed from six connexin molecules which extend a uniform distance outside the cells. Alignment of connexons from each cell across the gap results in the formation of the pores which functionally define the gap junction. Gap junctions are dynamic structures because connexons are able to open and close in response to the intracellular environments, e.g., elevated intracellular calcium and low intracellular pH are established stimuli for rapid closing of connexons. Gap junctions are abundant in cardiac and smooth muscle allowing electrical coupling between cells with depolarization of one group of muscle cells rapidly spreading to adjacent cells. Many hormones act by elevating intracellular concentrations of cyclic AMP, which initiates a signalling pathway inside the cell. Cyclic AMP readily passes through gap junctions and thus, hormonal stimulation of one cell can lead to signal propagation to a cluster of cells.

7.3.3 OCCLUDENS JUNCTIONS

Anchoring junctions provide the essential cell-cell and cell-matrix adhesion necessary for maintaining the integrity, and allowing the folding and bending, of epithelial cell sheets. Occludens junctions (zonula occludens or tight junctions) in contrast serve principally in generating the barrier properties of epithelial sheets. Tight junctions are the most apical of the intercellular junctions selectively restricting the intercellular diffusion of solutes and ions on the basis of solute molecular size, shape and charge, and in doing so minimizing the paracellula ("between cell") transfer of potentially harmful solutes and thus maximizing the functional significance of the cells' plasma membrane active transport systems. The tight junctional complexes also fulfill a role as a membrane "fence" restricting the inter-mixing of apical and basolateral membrane lipids and proteins. This confinement of specific proteins and lipids to specific membranes leads to the polarisation of the cell, i.e., the cell possesses two distinctively different membrane surfaces, i.e., the apical and basolateral domains, and, by inference, different capabilities for interacting with drug molecules. For example the drug efflux transporter P-glycoprotein (P-gp) is localized specifically to the apical (i.e., luminal) membranes of the renal proximal tubule, and small intestinal enterocyte, epithelial cells.

By the technique of freeze fracture electron microscopy the tight junctional complexes appear as a set of continuous, anastomosing intramembraneous strands which contact similar strands on the adjacent cells and thus seal the intercellular space (Figure 7.2A and Figure 7.2B). Figure 7.2C represents a schematic of the proteins involved in the transmembrane generation of extracellular tight junctional strands. Occludin (a protein of molecular weight (MW) approximately 60 kDa and comprising four transmembrane domains) is one of the major constituents of the tight junctional strands or fibrils. There is only one occludin gene although two isoforms can be generated by alternate splicing of the primary ribonucleic acid (RNA) transcript. Occludin was the first candidate protein for fulfilling the functional restrictive properties of the tight junctional fibril network.

More recently other proteins have been identified, including the claudin protein family, the members of which are integral membrane proteins localizing to tight junctional strands and which bind homotypically to claudins within the tight junctional strands of adjacent cells. To date, greater than 20 members of the claudin gene family have been identified. Their molecular weight is approximately 22 kDa and they comprise four transmembrane domains. The members of this family appear to show a differential pattern of tissue distribution, e.g., claudin-5 has been found only in endothelial cells. Current opinion is that the claudin family of proteins serve a central, but not the sole, role in generating the barrier properties of tight junctional complexes. Further, a junction-associated membrane protein (JAM) (molecular weight 40 kDa), a member of the immunoglobulin superfamily, has been localized to tight junctional complexes, although still little is known about the functional role of this protein.

In addition there are several accessory proteins localized to the cytoplasmic surface of tight junctions. The zonula occluden (ZO) proteins, ZO-1, ZO-2, and ZO-3 form heterodimers potentially serving as the major molecular scaffold for the tight junctional network. The ZO complexes (through ZO-1 or ZO-2) form



FIGURE 7.2 Representation of tight junctions (TJ) within mammalian cellular barriers. (A) Freeze fracture micrograph showing the fibril nature of the tight junctional proteins strands (occluden, claudins, etc.) typified within an intestinal epithelial enterocyte; (B) Conventional transmission electron micrograph showing (arrow) the electron-dense tight junctional plaque formed between two interconnecting brain endothelial cells; (C) Schematic of the major proteins associated with tight junctions. The TJ is embedded in a cholesterol-enriched region of the plasma membrane. Claudins within TJ strands bind homotypically to claudins on adjacent cells to produce the primary seal of the TJ. Occludin proteins function as dynamic regulatory proteins further restricting paracellular permeability, as do proteins such as junctional adhesion molecules (JAM). Zonula occludens proteins (ZO1 and ZO2) belong to a family of membrane associated guanylate kinase-like proteins {XE "MAGUK Membrane associated guanylate kinaselike proteins"} that serve as recognition proteins for tight junctional placement and as a support structure for signal transduction proteins. Cingulin is a myosin-like protein that binds preferentially to ZO proteins and connects the TJ proteins to the cell's cvtoskeleton.

crosslinks between the tight junctional strands and actin filaments. Cingulin is a double-stranded myosin-like protein that associates with the cytoplasmic face of the tight junctional complex and apparently directly with ZO proteins. Cingulin may function in linking the tight junctional strands with the actomyosin cytoskeleton.

The Ca^{2+} ion has a key role to play in the maintenance of tight junctional paracellular restrictiveness. Ca^{2+} ions act primarily on the extracellular side of the cell interacting with the extracellular element of E-cadherin, a critical cell–cell adhesion molecule in the zonula adherens junction that lies underneath the zonula occludens. Extracellular Ca^{2+} activates E-cadherin, which is then able to aggregate with other E-cadherin molecules on the same cell, an

arrangement that favors binding to E-cadherin of an adjacent cell. In the absence of Ca²⁺ E-cadherins are inactive and cell-cell adhesion is lost leading to functional impairment of the tight junctional complexes; the divalent ion chelator, ethylenediaminetetraacetic acid (EDTA), is often used in laboratory studies of drug transport to chelate free Ca^{2+} and disrupt the restrictive properties of the tight junctions. Ca²⁺ ions also promote binding of E-cadherin with intracellular located catenins which in turn binds to vinculin, and actinin and, indirectly, to the cytoskeleton of actin. The cytoskeleton appears to fulfill a key role in delivering signals from the adherens junctions to the tight junctions. For example, intercellular interactions between E-cadherin activates phospholipase C which then cleaves phosphatidyl 4,5-bisphosphate into inositol 1.4.5-trisphosphate and diacylglycerol and activates protein kinase C. Multiple phosphorylation steps follow leading to the assembly, and even in the disassembly, of the tight junctional strands and modulation in paracellular transport of markers such as mannitol, sucrose, and dextrans. Inhibitors of microfilaments and microtubules disrupt tight junctional formation.

For some recent reviews on tight junction biology and regulation the reader is directed to the following citations: Balda and Matter (2000);¹⁰ Cereijido et al. (2000);¹¹ Lapierre (2000);¹² and the Advanced Drug Review Theme series by Mrsny and Nusrat (2000).¹³

7.3.4 ENDOTHELIAL BARRIERS

Endothelium is the cellular lining of the blood vessels and consists of simple squamous epithelium. Endothelium plays a critical role in the mechanics of blood flow, the regulation of coagulation, leucocyte adhesion, angiogenesis and vascular smooth muscle cell growth, and also serves as a barrier to the transvascular movement of liquids and solutes; the transcapillary regulation of the plasma molecules, in particular proteins, is a critical process in regulating tissue-fluid balance. Exchange of interstitial liquid and solutes from blood to tissues occurs across the capillary circulation. At any time only 5% of the total blood volume is in the capillary circulation. Most cells in the body are no more distant than 40-80 µm from a capillary. Capillaries can be classified according to their endothelial cell structure, those with a continuous endothelium that form tight junctional intercellular complexes are the most common type, e.g., endothelium found in capillary beds of muscle, skin, lung, and brain. In particular the capillaries of the brain, which constitute the blood brain barrier, are highly restrictive in terms of paracellular drug transport limiting effective access to the brain to drugs which can tranverse the endothelial cell by a transcellular route. Endothelium with fenestrations (cytoplasmic pores, 80-100 nm in diameter, extending the full thickness of the cell) line the glomerular capillaries of the kidney and are also seen in the capillaries underlying the gastrointestinal mucosa and endocrine glands. The capillaries of the liver (sinusoids) possess a discontinuous endothelium with intercellular gaps which are often > 100 nm in diameter.

For review articles on microvascular structure and permeability refer to: Micheal and Curry (1999);¹⁴ Firth (2002);¹⁵ Stan (2002);¹⁶ and Feng et al. (2002).¹⁷

7.4 MEMBRANE TRANSPORT

7.4.1 MEMBRANE PERMEABILITY AND PASSIVE DIFFUSION

In considering absorption across an epithelial barrier then the rate at which drug molecules reach the systemic circulation will (assuming rate of drug release from the formulation is not rate-limiting) depend upon: (i) the rate of drug dosing which takes into account the administered dose (mass) and the dosing interval (t, time); (ii) the extent of oral bioavailability (F, %), and (iii) the apparent absorption rate constant for the drug (K_a , time⁻¹). The parameters F and K_a will themselves depend upon the rate and extent of release of drug from the formulation, the solubility of drug in the epithelial lining fluid, the surface area available for absorption, and the residence time of the drug in solution at the absorption site. Clearly also the stability of drug during the absorption process and importantly the intrinsic permeability of the epithelial barrier to the drug are critical factors in determining F and K_a .

It is the permeability of an absorption barrier that our discussion now focuses, with initial attention given to passive transport mechanisms. Passive diffusion involves the movement of drug molecules down a concentration or electrochemical gradient without the expenditure of energy. First, if we consider only the transport of drug molecules across an epithelial barrier via passive diffusional processes then the overall flux (J) of a drug in one dimension, i.e., the net mass of drug that diffuses through a unit area per unit time, can be described by Equation (7.1):

$$J = -DK_{\rm p}A\left(\frac{\mathrm{d}C}{\mathrm{d}x}\right)_t\tag{7.1}$$

where J is the flux of drug, D is the diffusion coefficient of drug across the cellular barrier, K_p is a global partition coefficient (cell membrane/aqueous fluid), A is the surface area of the barrier available for absorption, x is the thickness of the absorption barrier, and $(dC/dx)_t$ is the concentration gradient of drug across the absorption barrier. The negative sign in Equation (7.1) indicates that diffusion proceeds from high to low concentration and hence the flux is a positive quantity. The greater the concentration gradient, the greater the rate of diffusion of a drug across the cell membrane.

The apparent permeability coefficient (ρ) of an epithelial barrier to a given drug will approximate DK_p . The processes of drug partitioning with the cell (including partitioning between extracellular fluid and plasma membrane, partitioning between plasma membrane and cell cytosol, and other organelle interactions, etc.) and of drug diffusion across the cell (including a range of organelle and macromolecule interactions that will influence the diffusion

process) will themselves depend upon the molecular properties of the drug. These can be categorized as steric properties (i.e., molecular size, shape, volume), ionic properties (i.e., hydrogen bonding potential, pK_a) and hydrophobic properties. These molecular properties will determine if passive diffusional transport across an epithelial barrier will involve either a predominantly paracellular (between cells) pathway negotiating a tortuous intercellular route via the aqueous channels formed by the anastomosing tight junctional fibrils between adjacent cells, or predominantly (but not exclusively) transcellular (across the cell) pathway requiring partitioning of drug into the plasma membrane bilayer.

Most drugs are weak acids or weak bases, existing in aqueous solution as an equilibrium mixture of nonionized and ionized species. The nonionized species if sufficiently hydrophobic in nature will readily partition into cell membranes. In contrast, ionized compounds partition poorly and as a result will only be slowly transported across biological membranes. The ratio of nonionized to ionized drug when in aqueous solution depends upon the pK_a of the drug and the pH of the environment. Calculating the ratio of ionized to nonionized species can be determined by the Henderson–Hasselbach equation which can be written generically for either a weak base or weak acid as in Equation (7.2):

$$pH = pK_a + \log \frac{[\text{conjugate base}]}{[\text{conjugate acid}]}$$
(7.2)

If dealing with weak acid then the ionized species is the *conjugate base* and the nonionized is the *conjugate acid*. If dealing with a weak base then the ionized species is the *conjugate acid* and the nonionized is the *conjugate base*. For a weak acid then decreasing the pH below the molecule's pK_a will increase the proportion of nonionized species. Conversely, for a weak base the nonionized form predominates as the pH is increased above the pK_a of the molecule.

Plainly, if a drug's molecular properties afford partitioning into cellular membranes (i.e., nonionized form of the drug predominates and is of a sufficient hydrophobic nature) then the membrane surface area available for transcellular diffusion will be considerably greater, by many orders of magnitude, than the surface area available for diffusion via the paracellular route. As a corollary, transcellular diffusion will potentially lead to a higher epithelial permeability and a higher rate and extent of absorption. However, even for drugs displaying unfavorable membrane partitioning properties then their steric properties may nevertheless allow for a significant extent of absorption via the paracellular route, e.g., a linear polymer should display more favorable paracellular passage than a globular polymer of equivalent molecular weight.

The impact of the molecule's steric, ionic, and hydrophobic properties upon passive membrane transport and epithelial permeability are equally applicable for peptides, proteins, and nucleic acids, as they are for traditional low molecular weight drugs. Most peptides, proteins, and nucleic acids will display hydrophilic properties and possess good aqueous solubility, although the decapeptide immunosuppressant cyclosporin is an example of a very hydrophobic poorly water soluble peptide. Their permeation across biological barriers will be limited by the presence of a significant number of hydrogen bond acceptor and donor groups, i.e., requiring considerable desolvation energy for the molecule to leave the aqueous environment and partition into a biological membrane. Their diffusion across biological membranes, and indeed through the paracellular pathway will be slow; the diffusion coefficient of a drug is inversely proportional to its molecular weight, and while for traditional low molecular weight drugs (100-500 Da) the diffusion coefficient varies little between drugs, for biotechnology products this parameter can vary widely with the molecular weight biotechnology drugs from 1.2 kDa for the nonopeptide leuprolide through to $\sim 200 \text{ kDa}$ for full length IgG antibodies to many millions of Da for pDNA. Further, the epithelial permeability of biotechnology products will, like that of traditional low molecular drugs, be subject to the extent of degradation occurring within the barrier itself; for the former, the role of proteases and nucleases will be important.

7.4.2 FACILITATED PASSIVE DIFFUSION

Glucose, sodium ions and choride ions are just a few examples of molecules and ions that must efficiently get across the plasma membrane but to which the lipid bilayer of the membrane is virtually impermeable. Their transport must therefore be "facilitated" by proteins that span the membrane and provide an alternative transmembrane aqueous route for passage. Facilitated diffusion of certain solutes and ions can take place through transmembrane channels or carriers which span the lipid bilayer and enable the transport of solute or ion down its electrochemical (i.e., electrical charge and concentration) gradient (Figure 7.3). Because the driving force for the movement of the solute or ion is the electrochemical gradient of the transported molecule itself, then facilitated diffusion does not utilize energy. The facilitative transporters can, however, become saturated with substrate and thus unlike simple passive diffusion the rate of membrane transport via facilitative diffusion can display nonlinear kinetics. The carriers bind a specific solute and are thereby induced to undergo a conformational change which carries the solute to the other side of the membrane. The carrier then discharges the solute and, through another conformational change, reorients in the membrane to its original state. Typically, a given carrier will transport only a small group of related molecules. The channels are water-filled pores across the lipid bilayer, which are regulated, through the needs of the cell, to be in an open or closed position, e.g., ligand-, mechanically- or voltage-gated ion channels. The transport via a channel is usually significantly faster than that mediated by carriers. Ion channels allow currents to be carried across the membrane and are thus of particular importance in the physiology of excitable cells like neurons and muscle cells.



FIGURE 7.3 Schematic representation of the nature of membrane transporters. Molecules can traverse a membrane down their electrochemical gradiant by simple passive diffusion or if they lack the necessary membrane partitioning properties then they may gain access to the cell via facilitative transport mechanisms involving symporters or antiporters. Active transporter can move molecules against their electrochemical gradiant but require the direct expenditure of energy such as the hydrolysis of ATP.

A good example of facilitated transport is that for hexoses. As mentioned above hexoses (glucose, galactose and fructose) which serve as basic fuel molecules for the cell are unable to cross the plasma membrane by simple passive diffusion and require transporter proteins to gain access to the cell cytoplasm. Two distinct groups of hexose transporters have been identified: hexose carriers mediating facilitative diffusion (GLUT1, GLUT2, GLUT3, GLUT4, and GLUT5) and a hexose carrier (SGLUT1) mediating active transport, i.e., transport against a concentration gradient and requiring the expenditure of energy provided by an electrochemical gradient of sodium which is cotransported with the hexose, either glucose or galactose. GLUT1, 3 and 4 have a high affinity for glucose functioning at maximal rate under physiologic glucose concentrations. In contrast, GLUT2 has a low affinity for glucose which allows it to change transport rate in proportion to the increasing glucose concentrations that occur after ingestion of a carbohydrate-rich meal. The enterocyte possesses on its apical membrane contains the sodium-glucose cotransporter SGLUT1, which allows the cell to take up glucose and galactose by cotransport with sodium, and GLUT5, which mediates absorption of fructose. On the basolateral plasma membranes is GLUT2, which allows diffusion of all three of these hexoses out of the cell into extracellular fluid and ultimately, into blood.

7.4.3 ACTIVE CARRIER-MEDIATED TRANSPORTERS

Active transporters use energy to transport solutes against a concentration gradient, where the energy input is derived directly by the carrier from the

hydrolysis of ATP or indirectly from the energy stored in ion gradients, e.g., proton or more commonly sodium gradients. In the latter case of ion gradients the active transport of a solute against its electrochemical gradient is coupled to the transport of, for example, sodium, down its electrochemical gradient (Figure 7.3). The coupling of ion transport with that of the solute can either be of a symport type—where the ion and solute move in the same direction or of the antiport type—where the ion and solute move in opposing directions. A good example of a Na⁺-dependent symporter is the active glucose (SGLT) transporter mediating glucose and galactose uptake at the apical membrane of enterocytes. Other examples would be the amino acid transporters (system A and system ASC) expressed in a variety of epithelial membranes.

ATP-powered pumps (ATPases) couple the splitting, or hydrolysis, of ATP with the movement of ions across a membrane against a concentration gradient, where ATP is hydrolyzed directly to ADP and inorganic phosphate, and the energy released is used to move one or more ions across biological membranes. The Na⁺-K⁺ ATPase pumps Na⁺ outward, while it pumps K⁺ inward. This pump transfers three Na⁺ molecules to the outside of cell and brings in the two K⁺ instead. It is this movement of ions via the hydrolysis of ATP that indirectly drives symporter and antiporter function. As much as 25% of a cell's ATP reserves may be spent in ion transport.

7.4.4 NUCLEOSIDE TRANSPORTERS

Nucleoside transporters are required for permeation of nucleosides across biological membranes and are present in the plasma membranes of most cell types. Functional studies have established that nucleoside transport in mammalian cells occurs by two mechanistically distinct processes, facilitated diffusion and active Na(+)-nucleoside cotransport.

There are two families of human nucleoside transporters, concentrative (hCNTs) and equilibrative (hENTs). The members of both families mediate the transmembrane transport of natural nucleosides and some drugs whose structure is based on nucleosides. The human concentrative nucleoside transporters co-translocate nucleosides and sodium unidirectionally against the nucleoside concentration gradients. They are found predominantly in specialized tissues important for absorption (intestinal epithelia), distribution (blood-brain barrier), and elimination (hepatic and renal epithelia). These concentrative transporters show a high affinity for their natural substrates (with $K_{\rm m}$ values in the low micromolar range) and are substrate selective. The human equilibrative nucleoside transporters translocate nucleosides and nucleobases bidirectionally down their concentration gradients. These equilibrative transporters show a lower affinity and are more permissive regarding the substrates they accept. Their expression is more ubiquitous through the body. Both hENTs and hCNTs are tightly regulated both by endocrine and growth factors and by substrate availability. The degree of cell differentiation and the proliferation status of a cell also affect the pattern of expressed

transporters. Concentrative transporters are typically found in the apical membrane while equibrative transporters are typically found in the basolateral membrane.

The ENT family consist of two subtypes (hENT1 and hENT2), which are known to mediate the transport of purine and pyrimidine nucleosides down their concentration gradients. These transporters display different sensitivities to inhibition by nitrobenzylthioinosine, a nucleoside analog, nitrobenzylmercaptopurine ribonucleoside, i.e., hENT1 is equilibrative and sensitive (hENT1es) while hENT2 is equilibrative and insensitive (hENT1ei). The hENT1 is a ubiquitous transporter found in a diverse array of human tissues, in which it show significant contribution in relation to the uptake of nucleoside-mimic chemotherapeutic agents in addition to physiologic nucleosides. Despite hENT2 expression in a variety of tissues, its highest functional expression occurs in skeletal muscle where it possibly plays a predominant role as a transporter.

The hCNT1 is pyrimidine specific concentrative nucleoside transporter, while the hCNT2 is a purine specific concentrative nucleoside transporter. The CNT families are found to be distributed in highly differentiated tissues such as the epithelial lining of the intestine and kidney.

Both pyrimidine and purine nucleoside analogs are currently used clinically as antimetabolite drugs. Cytarabine, an analog of deoxycytidine $(1-\beta-D)$ -arabinofuranosylcytosine, araC, Cytosar-Us), is used as combination chemotherapy in the treatment of chronic myelogenous, leukemia, multiple myeloma, Hodgkin's lymphoma, and non-Hodgkin's lymphomas; gemcitabine (dFdC, 2',2'-di-uorodeoxycytidine, Gemzars), a broad spectrum agent, which is used for treatment of a variety of cancers including pancreatic and bladder cancers. Capecitabine (5'-deoxy-5-N-[(pentoxy) carbonyl]-cytidine, Xelodas) is used, as a prodrug, in treatment of metastatic colorectal cancer. Two purine nucleoside anti-metabolite drugs, fludarabine (9- β -D-arabinofuranosyl-2-uoroadenine), and cladribine (2-chloro-2'-deoxyadenosine, CdA, Leustatins) are used for treatment of low-grade lymphomas and chronic lymphocytic leukemia.

For further reading on mammalian cell membrane nucleoside transporters the reader is directed to the following reviews: Baldwin et al. (1999);¹⁸ Kong et al. (2004);¹⁹ Casado et al. (2002);²⁰ and Pennycooke et al. (2001).²¹

7.4.5 **PEPTIDE TRANSPORTERS**

Peptides are generally hydrophilic and often exhibit poor passive transcellular diffusion across biological barriers. Most of the mammalian peptide transporter proteins that have been identified belong to the proton-coupled oligopeptide transporter (POT) superfamily, the members of which transport various small di- and tripeptides and peptidemimetic-based drugs. In gastrointestinal tract ingested proteins are digested to small peptides that can be transported via POTs across the small intestine. While an electrochemical Na⁺ gradient mainly provides the energy for active transport of most organic

solutes, including amino acids and sugars, for the functioning of POTs it is a proton symport, driven by pH gradients, that is the coupling force for transport (Figure 7.3).

In mammalian cells the required proton gradient driving force is commonly provided by electroneutral proton/cation exchangers (e.g., Na^+/H^+ antiporters). The current model states that peptide or peptide-based drug uptake is coupled with the uptake of a proton into epithelial cells and is promoted by a POT peptide transporter(s) located in the apical membrane (e.g., hPepT1). Because protons are cotransported with peptides across the epithelial membrane, this system is also referred to as a proton-dependent peptide cotransport system. According to this model peptides that are not appreciably degraded intracellularly are transported out of the cells and across the basolateral membrane of other (unknown) basolateral peptide transporters.

So far two human peptide transporters have been cloned, namely hPept1 and hPept2. Recently, several new POT members, including peptide/histidine transporter 1 (PHT1) and peptide/histidine transporter 2 (PHT2) and their splice variants have been identified in human tissues of the intestine. This has led to an increased need for new experimental methods enabling better characterization of the biophysical and biochemical barriers and the role of these POT isoforms in mediating peptide-based drug transport.

Members of the POT superfamily vary in size from 450 to 790 amino acids and contain 12 predicted transmembrane α -helical domains, with a majority of the proteins having both the N- and C-termini localized intracellularly. There is significant sequence homology across various species for the Pep transporters, and the hPept1 and hPept2 isoforms share a 50% amino acid sequence homology with each other, but do not show a strong homology with any other cloned mammalian transporter proteins, (except Pep transporters in other species) including other H⁺-dependent transporters such as the folate transporter or the monocarboxylate transporter.

Considerable attention has been given to peptide transporter 1 (PepT1) as the main mammalian POT member regulating intestinal peptide absorption. The hPept1 in the adult is expressed in the brush border membrane of the epithelial enterocytes. However, hPept1 is also expressed in the liver, kidney, and pancreas. The hPepT2 is primarily found in the kidney localized to the apical membrane (i.e., facing the lumen of the nephron tubule) of the proximal renal epithelial cells where its role is considered to be in the active reabsorption of small oligo peptides. While greater than 50% of the plasma amino acid pool is in peptide-bound form (mostly as di- and/or tri-peptides). The concentration of small peptides in the renal tubular lumen is likely to be higher since larger peptides and small proteins filtered by the glomerulus will be subjected to hydrolysis by the highly active peptidases (e.g., dipeptidylpeptidase IV and aminopeptidase N) located within renal brush border membrane. The urinary excretion of acid-soluble, peptide-bound amino acids is very low, thus the renal peptide transporter system appears to serve an important function in the conservation of peptide-bound amino nitrogen. The hPepT2 has also been shown to be expressed in lung and brain tissue.

Although hPept1 and hPept2, display functional commonality they also show many differences in substrate specificity, affinity and also transport capacity. Kinetically, hPept1 is considered a low-affinity and high-capacity system with apparent affinity constants, $K_{\rm m}$, ranging from 0.2–10 mM, depending upon the substrate. However, hPept2 is considered as a highaffinity and low-capacity transport system showing $K_{\rm m}$ values of 5–500 μ M. These transporters do exhibit a relatively broad substrate specificity necessary for their biological function. The physiological substrates of hPept1 and hPept2 are different di- and tri-peptides; up to 400 different di-peptide or 8000 possible tri-peptide combinations are possible by the various permutations provided through the 20 natural L- α -amino acids. Although the peptide transporters are relatively stereoselective, showing greater affinity to peptides formed by L-isomer amino acids than those formed by D-isomer amino acids, peptides containing D-enantiomers at the N-terminal position nevertheless can display reasonably high affinities and be transported with fairly high rates. This is an indication that the substrate-binding sites provide some freedom to accommodate substrates possessing diverse structures. Not only do Pept1 and Pept2 transporters transfer a variety of chemically diverse di- and tripeptides but they also are able to transport peptidomimetic drugs including cephalosporins penicillins, and angiotensin converting enzyme inhibitors such as captopril.

The following articles may be consulted for further information on peptide transporters: Adibi (2003);²² Rubio-Aliaga and Daniel (2002);²³ Wright et al. (2003);²⁴ Ganapathy and Leibach (1996);²⁵ and Terada and Inue (2004).²⁶

7.4.6 AMINO ACID TRANSPORTERS

There are several transporters responsible for amino acid transport within mammalian cells. These transporters are part of one of the largest families of transporters, the amino acid/polyamine/choline {XE "APC amino acid/ polyamine/choline"} (APC) superfamily. The flow of amino acid nutrients into cells is mediated and regulated by amino acid transporters. Amino acids serve not only as a basic building block for proteins, but also serve functions in signal transduction, e.g., specific amino acids act as neurotransmitters, synaptic modulators, or neurotransmitter precursors.

Based upon transporter specificities to different amino acids, and the sodium-dependency of the transport systems, then amino acid transporters can be classified into different groups. These various transporter systems do, however, display significant substrate overlap for various amino acids although at different affinities. Essentially there are three major groups of amino acid transporters, namely neutral, cationic and anionic amino acid transporters, each displaying sodium-ion dependency and independency.

The neutral amino acid transport systems A, ASC, and L are ubiquitous in almost all cell types. The alanine-preference system (system A), and the alanine-, serine-, cysteine-preference system (system ASC) mediate the symport (with sodium) of amino acids with small side chains. System A actually favors alanine, serine, and glutamine, whereas system ASC favors alanine, serine, and cysteine. Transport of alanine can be inhibited (in the presence of sodium) by the model analog, *N*-methylaminoisobutyric acid (MeAIB).

Large neutral amino acids (LNAA) with bulky side chains (i.e., branched and aromatic groups) are transported bi-directionally in a sodium-independent manner via the leucine-preference system (system L) which possesses a high exchange property (i.e., it shows *trans*-stimulation, stimulation by substrates in the *trans*-compartment) and has been shown, in some circumstances, to mediate the efflux of amino acids from cells as well as their influx. Amino acids with a small, nonbranched side chain are poor substrates for system L. The analogues 2-aminoendobicyclo-[2,2,1]-heptane-2-carboxylic acid (BCH) and 3-aminoendobicyclo-[3,2,1]-octane-3-carbox-ylic acid (BCO) are model substrates for System L in the absence of sodium. System L is a heteromeric complex comprising light and heavy chain subunits. So far two subtypes of light chains (LAT1 and LAT2) are recognized, both interact with the same heavy chain, a Type-II glycoprotein termed 4F2hc. The LAT1 complex displays substrate affinity in the micromolar range, whereas LAT2 complex shows substrate affinity in the millimolar range.

Among cationic amino acid transporters the sodium-dependent system v^+ is the most widespread system in terms of tissue expression, whereas other systems (systems y^+L , b^+ , $b^{0,+}$, and $B^{0,+}$) display a more localized tissuespecific expression. System v^+ catalyzes the high-affinity (micromolar K_m) sodium-independent transport of cationic amino acids and also the transport of neutral amino acids with low affinity (millimolar $K_{\rm m}$). System y^+ is electrogenic and accumulates its substrates by coupling with the cell's plasma membrane potential. System y^+L seems to be specific to erythrocytes and the membranes of the placenta. System y^+ and y^+L activities can be discriminated by N-ethylmaleimide (NEM) treatment, i.e., being sensitive or resistant, respectively. The embryonic sodium-independent system b^+ shows the narrowest specificity of the cationic transporters restricting transport solely to cationic amino acids. Systems $B^{0,+}$ (sodium-dependent) and $b^{0,+}$ (sodium independent) display a much broader substrate specificity with high affinity (micromolar K_m) for cationic amino acids but also are able to transport small and large neutral amino acids. System $b^{0,+}$ displays preference for large unbranched neutral amino acids. Expression of the heavy chain subunit rBAT (a protein homologous to 4F2hc) is essential for system $b^{0,+}$ functionality.

Anionic amino acids such as L-glutamate and L-aspartate are shown to accumulate in many cells, e.g., neurons and glial cells, hepatocytes, enterocytes, fibroblasts, and placental trophoblasts, by the system X_{AG}^- which is a high affinity (micromolar K_m) sodium- and potassium-dependent anionic amino acid transporter. Both D- and L-stereo-isomers of aspartate can be transported by this system. Further, it is thought that all five glutamate transporters (EAAT 1–5) from the superfamily of sodium- and potassium-dependent transporters of anionic and neutral amino acids represent variants of system X_{AG}^- . In addition various cell types, e.g., hepatocytes, fibroblasts, and

embryonic cells, transport L-glutamate (specifically anionic amino acids with three or more carbon atoms in the side chain) and L-cystine (as tripolar ion) via the sodium-independent antiport system $X_{\rm C}^-$ (Km milimolar), which is not sensitive to membrane potential and also presents an example of a transporter with exchange functionality. It is believed that $X_{\rm C}^-$ participates in a glutamine-cystine cycle that helps cells to resist oxidative stress; that is, glutamine entering the cell via systems ASC and A is converted to glutamate, which is exchanged for cystine via the oxidative stress-induced $X_{\rm C}^-$. The accumulated cystine then supports glutathione synthesis which protects cells against oxidative stresses.

For review articles on the nature and functioning of amino acid transporters the reader is referred to the following: Hyde et al. (2003);²⁷ Verrey (2003);²⁸ Mann et al. (2003);²⁹ Closs (2002);³⁰ and Chilleron et al. (2001).³¹

7.4.7 EFFLUX TRANSPORTERS

The permeability of an epithelial or endothelial barrier to a drug is also subject to membrane transporters which recognize drugs as substrates and serve to restrict drug transport across the barrier. These transporters have been termed "efflux" transporters and the most documented belong to the ABC superfamily of transporters, including P-glycoprotein (P-gp) multidrug resistance proteins (MRPs) and breast cancer resistance protein (BCRP).

P-glycoprotein (P-gp) consists of two very similar halves, each containing six transmembrane domains and an intracellular ATP binding site. In humans there are two gene products MDR-1 and MDR-3 while in rodents there are three, i.e., mdr-1a and mdr-1b, which functionally correlate with the human MDR1, and the mdr-2, which functionally correlates with the human MDR3. The MDR-3 gene product has been shown to transport membrane components such as phosphatidylcholine, while MDR-1 is associated with the transport of a wide variety of xenobiotics including, among others, HIV protease inhibitors, corticosteroids, antibiotics and a range of cytotoxic agents, and as such contributes to the multidrug resistance (MDR) phenotype in cancer. P-gp MDR-1 is constitutively expressed at high levels in the bile cannicular membrane of hepatocytes and the villus tips of the enterocytes of the gastrointestinal tract. In this way xenobiotics may be extruded from blood into bile for excretion into the gastro-intestinal tract, and respectively, prevented from crossing the gastro-intestinal epithelium to be absorbed into the mesenteric blood supply. P-gp MDR-1 is also highly expressed within the luminal capillary membranes of the brain microvascular cells that constitute the bloodbrain-barrier. Additionally P-gp is expressed at the blood-testis barrier, within the apical (luminal) membranes of renal proximal tubule epithelial cells, and within apical (luminal) membranes of lung epithelial cells (both bronchiolar and alveolar). As such the constitutive expression of P-gp represents a significant barrier in epithelial and endothelial drug transport.

The issue of P-gp serving as a barrier to the absorption and tissue distribution of drugs is further compounded for the pharmaceutical industry by the fact that P-gp displays an enormous diversity in the structure of the substrates that it transports. Substrates for P-gp vary in size from 150 Da to approximately 2000 Da, many contain aromatic groups although nonaromatic linear or circular molecules are also transported. Peptide structures are also recognized as P-gp substrates. Many of the substrates that are most efficiently transported are uncharged or weakly basic in nature, but acidic compounds can also be transported, although generally at a lower rate. It is this wide diversity in substrate structure that has made it difficult to generate structure-activity-relationships (SAR) for P-gp. A common physicochemical property of P-gp substrates is however their tendency to be hydrophobic in nature, consistant with the fact that the P-gp substrate binding sites are buried within the lipid bilayer.

The family of multidrug resistance proteins (MRPs) includes to date MRP-1 through -9, with MRP-1 and MRP-2 being the most investigated with respect to pharmaceutical barriers. The MRP-4 and -5 proteins possess a similar structure to that of P-gp, whereas MRP-1, -2 and -3 possess an additional N-linked segment comprising a five transmembrane domain.

MRP1 displays quite a striking overlap with P-gp in respect to the transport of cytotoxic agents conferring resistance to antracyclines, vinca alkaloids, epipodophyllotoxins, camptothecins, and methotrexate, but not to taxanes which are an important component of the P-gp anticancer substrate profile. However, the general substrate selectivities of P-gp and MRP1 do nevertheless show marked differences in that while P-gp substrates are neutral or weakly positively charged lipophilic compounds, MRP1 is able to transport lipophilic anions including a structurally diverse group of molecules conjugated with glutathione, glucuronide or sulphate. Further, it appears that transport of neutral compounds such as some of the anticancer agents by MRP1 are also highly dependent upon cellular glutathione, with drug export involving a combination of co-transport with glutathione. As such MRP1-conferred resistance will be subject to inhibition with agents that block glutathione synthesis.

In epithelial cells MRP1 is localized on the basolateral membrane, and is expressed in the choroid epithelium effluxing substrate from cerebral spinal fluid (CSF) to blood. It is also expressed at high levels within intestinal mucosa, lung mucosa, and within the kidney. Low expression is seen in the liver.

MRP-2 (also termed cMOAT) like MRP-1 is essentially an organic anion transporter displaying similar substrate selectivity with respect to glutathione and glucuronide conjugates. The pattern of expression compared to MRP1 is, however, different with MRP2 expressed in the apical membranes of polarized cells, with particularly high expression in the liver cannaliculi, and with lower levels in the renal proximal tubules and intestinal enterocytes.

The substrate selectivity of MRP3 appears to overlap with that of MRP1 and MRP2 with respect to the transport of glutathione and glucuronide conjugates, although the affinity for conjugates is reported to be less than that

of MRP1 and MRP2, and its spectrum of anticancer agents transported more limited, and may not require glutathione for cotransport. MRP3 is expressed in the liver, intestine, adrenal gland and to a lower extent in the pancreas and kidney. Like MRP1, MRP3 is expressed on the basalateral membranes of epithelia.

MRP4 and MRP5 are organic anion transporters with the capacity to transport substrates such as oestradiol-17- β -glucuronide, methotrexate and reduced folates. In addition MRP4 and MRP5 are able to mediate the transport of cAMP and cGMP, with the ability to confer resistance to certain nucleotide analogs, for example 6-mercaptopurine, 6-thioguanine, and azidothymidine. MRP4 has been reported to be expressed in a number of tissues including lung, kidney, and small intestine among others. These can be found in either apical or basolateral membranes depending upon the tissue in question. MRP5 shows highest expression in the brain and skeletal muscle and is expressed in the basolateral membranes.

Breast cancer resistant protein (BCRP) was first cloned from a doxorubicin-resistant breast cancer cell line. However, its expression is not specific for breast cancer cells and indeed this transporter may not necessarily fulfill a critical role in chemotherapy resistance in breast cancer. BCRP can be considered structurally as a half-transporter comprising only of a single six-transmembrane domain segment with a single N-terminal ATP binding site. In polarized epithelial cells BCRP localizes to the apical membrane. It is expressed in the kidney, in the bile cannalicular membrane of liver hepatocytes, and the luminal membrane of the epithelial cells of the small and large intestines. BCRP is also found in apical surface of the ducts and lobules in the breast, and in many microvascular beds.

There is considerable but varying overlap in the anticancer drug substrate specificity between BCRP, P-gp, MRP1, and MRP2, with likely substrates including mitoxantrone, topptecan, and doxorubicin. Little or no resistance is seen for vincristine, paclitaxel or cisplatin.

An area of research that has attracted much attention within the pharmaceutical industry is the identification of P-gp inhibitors that could be used in the management of cytotoxic resistant tumors expressing P-gp MDR-1. The second- and third-generation inhibitors have been specifically designed to inhibit P-gp with associated low toxicities and high selectivities. PSC833 (valspodar) is a structural analog of cyclosporine A (a clinically effective immunosuppressant and first- generation P-gp inhibitor) that also inhibits MRP-2. Another third-generation inhibitor, GF120918 (elacridar) inhibits both P-gp and BCRP, while LY335979 (zosuquidar) is able to inhibit P-gp without apparent effects upon MRP-1, MRP-2, MRP-3, or BCRP. For both valspodar and zosuquidar produce effective P-gp inhibition achievable without effect upon CYP3A4.

The reader is referred to the following review articles on ABC efflux transporters: Chang (2003);³² Kruth and Belinsky (2003);³³ Leonard et al. (2002),³⁴ and the Advanced Drug Reviews theme issue on drug efflux transporters edited by Miller (2001).³⁵

7.4.8 PHARMACOGENOMICS OF MEMBRANE TRANSPORTERS

Pharmacogenomics is concerned with the impact of genetic variation upon an individual's response to a drug. Polymorphisms are allelic variants in genes that exist stably in the population, conventionally with an allele frequency above 1%. Genetic polymorphisms in drug transporters may affect drug absorption, distribution and elimination. A human membrane transporter database has been established that contains information on the genetics of human membrane drug transporters, including any polymorphisms that have been identified (Yan and Saddee, (2000);³⁶ http://lab.digibench.net/transporter/). A SNP database can also be located at http://www.ncbi.nlm. nih.gov/SNP/index.html.

7.4.9 NUCLEAR MEMBRANE TRANSPORT

For strategies involving plasmid DNA and for sequences of antisense oligonucleotide binding that are designed to interact with nascent RNA, then the nucleus is the target subcellular organelle. Except when cell division occurs any nuclear-cytoplasmic exchange must take place across an intact nuclear membrane envelope. In the absence of cell division the exchange of macromolecules between nucleus and cytoplasm across an intact nuclear membrane occurs through nuclear pore complexes (NPC). The nuclear envelope of a typical mammalian cell consists of inner and outer membrane layers perforated by 3000 to 4000 nuclear pores. The nuclear pore complex itself (Figure 7.4) is an aqueous channel surrounded by large protein granules arranged in an octagonal array. It contains up to 100 different proteins with an external diameter of 120 nm. The aqueous pore itself is blocked by a central granule which can vary in shape and size with diameters ranging from 2.5 nm to 35 nm; a number of fibrils emanate from this central granule. The granule is involved in both limiting passive cytoplasmic-nucleoplasmic transport and also in the process of active transport of molecules into the nucleus from the cytoplasm. By determination of diffusion coefficients an aqueous channel (~ 10 nm in diameter) within the nuclear pore complex is predicted that allows passive transport through the nuclear pore complex of molecules of appropriate dimensions (corresponding to approximately <20-40 kDa); albumin (molecular weight 60 kDa is excluded from passive nuclear membrane transport. Clearly many large cellular protein complexes must gain access to the nucleus (nucleardestined proteins are termed karyophilic proteins), and hence a regulated active transport process through the nuclear pores must also be functional.

Cytoplasmic proteins are selected for active nuclear import by the recognition of specific sequences within their structures. These sequences are known as nuclear localization signals (NLS). NLS are short peptides consisting predominately of the highly basic residues lysine and arginine. The most extensively characterized NLS is that contained within the SV40 large T antigen; this protein is essential for the viral replication of SV40 DNA within the host nucleus. The active transport of an NLS-containing protein involves



FIGURE 7.4 Illustrative diagram of active nuclear membrane transport. Cytoplasmic proteins are selected for active nuclear import by the recognition of specific sequences, nuclear localization signals (NLS), within their structures. In order for the NLS-protein to bind to the nuclear pore complex it must firstly bind to the α and β subunits of karyopherin (also known as importins). Nuclear import requires the presence of the GTPase, Ran (in its GDP-bound state) and the nucleoporin docking protein, p10. These soluble factors are involved in a series of association–dissociation steps, which move the NLS-protein/karyopherin α/β heterotrimer towards the nucleoplasmic side of the pore complex. The α -subunit of karyopherin then catalyzes the transformation of Ran into its GTP-bound state. This complex is actively transported into the nucleoplasm, leaving the β -subunit bound to the pore. Once inside the nucleus the α -subunit complex dissociates.

two steps. The first step is an energy-independent binding of the nuclear pore complex. This is followed by a GTP-dependent translocation across the nuclear pore into the nucleus. Both these processes require the presence of soluble factors. In order for the NLS-protein to bind to the nuclear pore complex it must first bind to the α and β subunits of karyopherin (also known as importin). The NLS-binding protein, karyopherin α , forms a weak complex with the NLS-protein in the cytoplasm. This dimer is stabilized by the presence of karyopherin β , which also promotes interaction with the proteins of the nuclear pore complex (nucleoporins). The translocation step of nuclear import requires the presence of the GTPase, Ran (in its GDP-bound state) and the nucleoporin docking protein, p10. These soluble factors are involved in a series of association-dissociation steps, which move the NLS-protein/karyopherin α/β heterotrimer towards the nucleoplasmic side of the pore complex. The α -subunit of karyopherin then catalyzes the transformation of Ran into its GTP-bound state. This change causes the dissociation of p10 from the GTPase which in turn dissociates the karyopherin α /NLS-protein complex from karyopherin β . As a result the complex is free to move into the nucleoplasm, leaving the β -subunit bound to the pore. Once inside the nucleus the α -subunit complex dissociates, possibly due to the differential ionic conditions of the nucleoplasm or alternatively by interaction with other nuclear factors, freeing the nuclear protein.

An interesting feature of the NLS is that synthetic peptides that contain NLS are capable of functioning as transport signals when chemically conjugated with a non-nuclear destined proteins, and more significantly to plasmid DNA and DNA delivery vectors. The use of NLS in gene therapy vectors is encouraging and further supports the development of gene therapy vectors that mimic viruses.

For further reading the reader is referred to: Fahrenkrog and Aebi (2003);³⁷ Fried and Kutay (2003);³⁸ Bednenko et al. (2003);³⁹ and the Advanced Drug Reviews theme issue on nuclear transport edited by Dean (2003).⁴⁰

7.5 VESICULAR TRANSPORT

Most pharmaceutical scientists will have used the terms endocytosis and transcytosis to describe events leading to the internalization and trans-barrier transport of macromolecules. In this section we will describe the dynamics involved in the intracellular movement of membrane-enclosed compartments or vesicles, and some of the molecular mechanisms underlying the fission (i.e., budding) and fusion of these vesicles with other membranes.

Eukaryotic cells possess an internal membrane system that allows them to internalize macromolecules from their environment, i.e., the process of endocytosis, and to shuttle these internalized molecules between chemically distinct membrane-enclosed vesicles to afford their metabolic utilization. This internal membrane system also provides a biosynthetic-secretory pathway by which cells can sequentially, and in a temporally-regulated manner, modify biosynthesized proteins during transit from the endoplasmic reticulum through the Golgi apparatus en route to specific cell surface domains; secretion of these proteins to the exterior is by a process termed exocytosis. Through specific and regulated mechanisms these compartments or vesicles undergo fusion with, and fission from, each other to exchange the cargo that they bear. Shown in Figure 7.5 is an outline of the endocytic and secretory pathways which ultimately provide connection between the endoplasmic reticulum and the plasma membrane.

Many of these transport vesicles bud from membrane domains rich in specialized coat proteins which appear important for the domain localization of specific cell surface receptors and also signalling molecules, and also for imparting biophysical aspects to these membrane domains that promote the budding and vesicle formation. The three most characterized protein coats surrounding transport vesicles are clathrin, COPI subunits and COPII subunits, which respectively give rise to clathrin-coated vesicles mediating transport from the trans-Golgi and from the plasma membrane, and COPI and



Rough endoplasmic reticulum

FIGURE 7.5 Schematic diagram of the intracellular membrane vesicles that constitute the endocytic and biosynthetic-secretory pathways of a cell. Endocytosis at the plasma membrane involves the invagination and budding of a membrane domain, e.g., clathrincoated pit. The resulting trafficking vesicle is directed toward an early endosome compartment which serves as a sorting station. In the case of clathrin coated vesicles, the clathrin protein coat is lost very quickly after budding from the plasma membrane. From the early endosome endocytosed material can be retrieved for recycling back to the original membrane (e.g., apical) or for transport to an opposing membrane (e.g., basolateral), or such material may be shuttled to a late endosome on-route to the lysosome for degradation. The biosynthetic pathway begins with protein synthesis at the endoplasmic reticulum (ER) and subsequent vesicular trafficking from the ER to the cis-Golgi (COPII-coated vesicles), then through the Golgi-cisternae (COPI-coated vesicles) where sequential post-translational modifications of the protein can take place. The protein is then passed through the trans-Golgi on-route to the plasma membrane or directed to the late endosomal/lysosomal compartments.

COPII coated vesicles which mediate transport between the endoplasmic reticulum and the Golgi cisternae; COPII vesicles budding from the endoplasmic reticulum and COPI vesicles from Golgi cisternae. Other vesicle types would include caveolae, which are a form of lipid raft and bear as an important structural and functional coat protein, caveolin. There undoubtedly are also other types of transport vesicles and budding membrane domains whose protein coats are yet to be fully characterized.

7.5.1 ENDOCYTOSIS

Endocytosis is subdivided into the processes of phagocytosis and pinocytosis. Phagocytosis is the ingestion of relatively large particulates, e.g., microrganisms, by specialized cell types that firstly adhere to the particulate and then generate membrane extensions (pseudopodium) around an adhered particle, leading to the eventual membrane fusion of these pseudopodium to enclose the particle within an internal membrane bound compartment termed a phagosome. Internal fusion of the phagosome with a lysosome then generates a phagolysosome within which the particle is acted upon by the engagement of a range of destructive processes including proteases, nucleases, reactive oxygen species, etc. Phagocytic cells would include macrophages, neutrophils, and dendritic cells.

Essentially all cells undertake pincytosis which refers to the internalization or invagination (as opposed to the generation of membrane pseudopodium) of plasma membrane domains, and with the invagination and internal budding of such domains from the cell surface then any bound molecules and associated extracellular fluid are also taken into the cell. Pinocytosis begins at specialized regions or domains of the plasma membrane including clathrin-coated pits, non-coated lipid rafts and caveolae. The invaginated plasma membrane gives rise to membrane-enclosed vesicles within the cytoplasm termed endosomes. From here the endosomes can undergo various trafficking routes as depicted in Figure 7.5. All cells will continually or constitutively internalize plasma membrane by this pinocytosis mechanism with, for example, fibroblasts internalizing approximately 1% of the plasma membrane surface every minute. To maintain cell volume and cell surface area then the loss of internalized plasma membrane arising from pinocytosis must be balanced by return of membrane to the cell surface; a balance achieved through by exocytosis, i.e., the fusion of internal vesicles with the plasma membrane resulting in the extrusion of material into the extracellular fluid.

7.5.2 CLATHRIN-MEDIATED ENDOCYTOSIS

Clathrin-mediated endocytosis is the most studied of the endocytic processes. Clathrin-coated pits (diameter of approximately 200 nm) can occupy as much as 2% of the total plasma membrane and at the cytoplasmic face of this domain is found the major coat protein, clathrin, which under the electron microscope can be seen as an electron-dense coat (Figure 7.6B). A clathrin subunit consists of three heavy (180 kDa) and three light (40 kDa) chains and together these form a three-legged structure called a triskelion (Figure 7.6C). Clathrin triskelions assemble into a basket-like convex framework (Figure 7.6C) providing an important geometry for the formation of the clathrin-coated pit and helping to stabilize the vesicle as it buds from the membrane.

Multiple protein-lipid and protein-protein interactions are involved in the recruitment of coat proteins to a membrane. Adaptin is a multisubunit protein



FIGURE 7.6 (A) Multiple protein–lipid and protein–protein interactions are involved in the recruitment of proteins to a clathrin-coated pit. Adaptin-2 (AP-2) binds to particular plasma membrane receptor and to each clathrin heavy chain and in doing so promotes the polymerization of the clathrin subunits, the recruitment of a particular set of receptor membrane proteins into clathrin-coated pits (Step 1. Coat assembly and cargo selection), and ultimately the invagination of the pit (Step 2. Bud formation). The budding of a clathrin-coated pit to form a vesicle requires a cytosolic GTPase, dynamin (Step 3. Vesicle formation). After entering the cytoplasm the endocytic vesicle rapidly sheds its clathrin coat through the actions of a uncoating ATPase (heat shock protein Hsp70) and then trafficks to early endosomes which are cellular compartments proximal to the plasma membrane (Step 4. Uncoating). (B) Electron-micrograph showing an electron-dense clathrin-coated pit (arrow) at a cell's plasma membrane. (C) A clathrin subunit consists of three heavy (180 kDa) and three light (40 kDa) chains and together these form a three-legged structure called a triskelion. Clathrin triskelions assemble into a basket-like convex framework.

complex within clathrin-coated pits located at the cytoplasmic face of the plasma membrane. Adaptin binds to particular plasma membrane receptors, for example epidermal growth factor receptor, via specific signal sequences within the carboxy terminus of the receptor. Another part of the adaptin

molecule then binds to the globular domain at the end of each clathrin heavy chain and in doing so promotes the polymerization of the clathrin subunits, the recruitment of a particular set of receptor membrane proteins into clathrincoated pits and ultimately the budding of the pit to form an endosome (Figure 7.6A). The adaptins that work at the cell surface are adaptor protein-2 (AP-2). A neuronal adaptor protein AP180 and its non-neuronal isoform CALM are monomeric proteins with binding sites for both clathrin and AP-2. These AP180 family members also bind to phosphatidylinositol containing phospholipids which have an important role in clathrin-coated pit formation, in particular controlling the targeting of AP2 to specific membrane domains. AP-2 and AP180 work synergistically to promote clathrin assembly at the appropriate target membrane. Protein coat assembly must be regulated in a spatial and temporal manner, and members of the family of monomeric GTPases serve as coat-recruitment proteins. For example ADP-ribosylation factors (ARF) proteins are responsible for COPI and clathrin coat assembly at the Golgi apparatus, while surface area regulation I (SarI) is responsible for COPII coat assembly at the endoplasmic reticulum.

The budding or fission of a clathrin-coated pit from the plasma membrane is a regulated process and an important component of this event is dynamin, a cytosolic GTPase (generating energy by triggering the hydrolysis of GTP to GDP), which serves either as a molecular switch or as a mechanochemical enzyme. Dynamin, which actually comprises an expanding family of proteins, assembles into oligomers at the neck of a budding vesicle (Figure 7.6A), and then recruits other proteins to this site, e.g., amphiphysin and endophilin. This complex at the neck of a budding vesicle distorts the bilayer to bring the two noncytosolic membrane leaflets into close proximity to one another leading to membrane fusion and the sealing off of the vesicle from the plasma membrane. After entering the cytoplasm the endocytic vesicle rapidly sheds its clathrin coat through the actions of a uncoating ATPase (heat shock protein Hsp70) and then trafficks to early endosomes which are cellular compartments proximal to the plasma membrane, and which receive endocytosed material and sort this material for further vesicular transport to late endosomes or for recycling to the plasma membrane.

Cells will endocytose material from their extracellular environment through one of the following processes: (i) receptor-mediated endocytosis, where a macromolecule will bind to its cognate cell membrane receptor, and the receptor-ligand pair is then internalized through a plasma membrane invagination such as a clathrin-coated pit to form an endocytic vesicle. Broadly, receptor-mediated endocytosis can be considered as constitutive (Class I) or ligand-stimulated (Class II). Constitutive endocytosis will result from the continual plasma membrane turnover and a receptor within a clathrin-coated pit can be internalized within an endocytic vesicle even in the absence of ligand. This constitutive internalization occurs for receptors for transferrin (the iron carrying plasma protein) and for low density lipoprotein. In ligand-stimulated endocytosis the ligand binding to its receptor triggers the internalization. Examples of this include inulin and epidermal growth factor binding to their respective receptors. With receptor-mediated endocytosis the rapid rates of internalization are possible but the receptor sites can be saturated at high ligand concentration; (ii) adsorptive endocytosis, where the material that is to be endocytosed binds to the cell surface not through a specific receptor-ligand pairing but through nonspecific mechanisms, e.g., electrostatic interactions. The adsorption and subsequent endocytosis of cationic lipidic or polymeric gene complexes to the anionic cell surface is an example of such an adsorptive endocytosis process. Saturation of non-specific membrane binding sites is less likely than with the receptor-mediated process; (iii) fluid-phase endocytosis, where the material to be endocytosed is simply present within the extracellular fluid bathing the cell surface, and as the plasma membrane invaginates to form an endocytic vesicle, either constitutively or by a ligandstimulated event, then some of the extracellular fluid is captured within the lumen of the budding vesicle. An example of this is the endocytosis of horseradish peroxidase or of dextrans which are commonly referred to as fluidphase markers. This is often associated with the slowest rates of internalization, and with the rate increasing linearly with concentration of the material in the extracellular fluid.

7.5.3 TARGETING AND FUSION OF VESICLES

The targeting and fusion of an endocytic vesicle with other vesicles (Figure 7.7), such as early endosomes, has to be regulated to ensure appropriate target delivery, and is mediated mainly through two classes of proteins: SNAREs and the targeting GTPases, Rabs. Broadly, SNARE proteins provide specificity in vesicle–vesicle interaction and in catalyzing the fusion of vesicle to target membrane. Rabs interact with other proteins to regulate the initial docking and tethering of the vesicle to specific target membrane.

There are at least 20 different SNARE proteins each associated with a particular membrane vesicle or membrane-enclosed organelle in the biosynthetic or endocytic pathways. These SNAREs display complementarity in that they exist as vesicle-membrane SNARE (v-SNAREs) and target-membrane SNARE (t-SNAREs) pairs each with characteristic helical domains. When a particular v-SNARE interacts with a particular t-SNARE then the helical domains interact to form a stable SNARE complex locking the two membranes together. After membrane fusion then the v- and t-SNAREs remain complexed in the fused membrane, and as such before these SNAREs can participate in another round of membrane interactions they have to be untangled by the action of a cytosolic ATPase termed N-ethylmaleimide-sensitive factor (NSF), which also uses soluble NSF attachment protein (SNAP) as a co-factor. Other inhibitory proteins exist, associated with the untangled SNAREs, which serve to limit indiscriminate SNARE interactions, e.g., the inhibitory LMA-1 associated with untangled t-SNAREs (Figure 7.7).

Rab proteins cycle between the cytosol and the membrane and provide specificity in vesicular fusions, facilitating and regulating the rate of vesicle docking and the pairing of v- and t-SNARES. Rabs are GTPases with over 30



FIGURE 7.7 Schematic showing the targeting and fusion of an endocytic vesicle with another membrane. Membrane fusion is mediated mainly through two classes of proteins: SNAREs and the targeting GTPases, Rabs. SNAREs display complementarity in existing as vesicle-membrane SNARE (v-SNAREs) and target-membrane SNARE (t-SNAREs) pairs each with characteristic helical domains. When a particular v-SNARE interacts with a particular t-SNARE then the helical domains interact to form a stable SNARE complex locking the two membranes together. Rab proteins cycle between the cytosol and the membrane and provide specificity in vesicular fusions, facilitating and regulating the rate of vesicle docking and the pairing of v- and t-SNARES. In the cytoplasm the Rab proteins are inactive (GDP is bound), while at the membrane in their GTP-bound state they are active. After membrane fusion then the v- and t-SNAREs remain complexed in the fused membrane, and as such they have to be untangled before they can enter into further rounds of docking and fusion.

identified members. Each Rab protein has a characteristic distribution on cell membranes with all membrane vesicles or membrane-enclosed organelles bearing on the cytoplasmic face at least one Rab protein. For example: Rab4 associated with early endosomes; Rab5A associated with plasma membrane and clathrin-coated vesicles; Rab5C associated with early endosomes; Rab7 associated with late endosomes; and Rab9 associated with late endosomes and

trans-Golgi network. In the cytoplasm the Rab proteins are inactive (GDP is bound), while at the membrane in their GTP-bound state they are active. On the membrane the Rab proteins interact with other protein effectors to facilitate the membrane docking process.

7.5.4 ENDOSOMES AND INTRACELLULAR VESICLES

Early endosomes form a compartment that acts as an early sorting station in the endocytic pathway. Endocytosed molecules will traffick to early endosomes within 5 to 10 min of vesicle budding from the plasma membrane. A biochemical marker for early endosomes is early endosome antigen-1 (EEA-1) which functions in the fusion of endosomes and appears to serve as a Rab5 effector binding phosphatidylinositol-3-phosphate (a product of phosphatidylinositol-3-OH kinase), an important component for endocytosis in its own right. Early endosomes have an intra-vesicular pH of 5.9 to 6.0 and in this acidic pH many internalized receptor proteins change their conformation and release their ligand. Those endocytosed ligands that dissociate from their receptor in the early endosome will be destined for ultimate delivery to lysosomes for destruction, e.g., Low density lipoprotein (LDL) dissociates from its receptor within the early endosome, with the resultant delivery of LDL to lysosomes. Delivery to lysosomes will also clearly be the case for molecules that have been internalized by fluid-phase endocytosis.

Some endocytosed ligands, however, remain bound to their receptor within the early endosome. From here these ligand bound receptors may be trafficked to other parts of the cell. For example, receptors retaining the binding of their ligand within the early endosome may return to the same plasma membrane domain from which they originally came. A good example of this is the transferrin receptor and its ligand, the plasma protein transferrin. Transferrin binds iron in the blood (iron bound transferrin is termed halo-transferrin) and in this state the halo-transferrin will have (at pH 7.4) a higher affinity to bind to the transferrin receptor. Once internalized via receptor–mediated endocytosis to early endosomes the low pH conditions induce halo-transferrin to release its bound iron (termed apotransferrin) remains attached to the transferrin receptor. The receptor–apotransferrin complex is recycled to the original plasma membrane domain from whence it came, e.g., apical domain, and at the extracellular pH of 7.4 the apotransferrin dissociates.

Both the receptor and the retained bound ligand may together be trafficked from early endosomes to lysosomes for degradation. This is the situation for epidermal growth factor (EGF) receptor and its ligand EGF. EGF binding to its receptor activates a signalling cascade stimulating kinases within the mitogen activated pathways leading to cell proliferation. Following activation of signal transduction the EGF receptor with bound ligand is delivered to lysosomes for destruction. From the early endosome ligand bound receptors may also enter a transcytotic pathway which ultimately results in delivery of ligand to a different plasma membrane domain, e.g., ligand has been trafficked from apical to basolateral plasma membrane domains. In peripheral microvasculature albumin appears to undergo a transcytotic trafficking route following binding to its receptor, gp60. Similarly, the neonatal receptor for Fc domains of IgG immunoglobulins (FcRn) also appears to traffick across polarized cells of the intestinal epithelium and also the bronchial epithelium.

Unless endocytosed material is selectively retrieved from early endosomes it is delivered via transport vesicles through multivesicular bodies to late endosomes which will eventually mature into lysosomes. Late endosomes possess a pH of about 5.5 to 6.0 and display as a marker for the mannose 6-phosphate (M6P) receptor which serves as a receptor to translocate lysosomal hydrolases (which bear on the termini of their N-linked oligosaccharide chains mannose-6-phosphate) from the trans Golgi network to late endosomes in route to the lysosome. As the pH drops within late endosomes during vesicle maturation into lysosomes hydrolases within the late endosome begin to degrade endocytosed material. Unless the endocytosed material is selectively retrieved to be transported to the Golgi it will then be exposed to the full hydrolytic activity of lysosomes which have formed from maturation of the late endosomes. Lysosomes contain in excess of 40 hydrolytic enzymes, including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases, and sulfatases. The pH of lysomes is 5.0, a pH optimal for the function of the acid hydrolases. Lysosomes are negative for M6P receptor but bear as a marker lysosome associated membrane protein 1 (Lamp-1).

In polarized cells endocytosis will occur from both apical and basolateral plasma membrane surfaces of the cell. These distinct cell faces possess their own early endosomal compartments unique to that respective domain. This arrangement allows endocytosed receptors to be recycled back to their original membrane unless they possess signals allowing them to be trafficked by transcytosis. Molecules not retrieved from either the apical or basolateral early endosome compartments are delivered to common late endosomes that are not domain unique.

7.5.5 ENDOSOMES AND THE CYTOSKELETON

The transport vesicles move along the cell's cytoskeletal structures which include: (i) the microtubule network comprising fibres composed of subunits of tubulin termed α -tubulin and β -tubulin. This is the main cytoskeletal element driving vesicle movement with motor proteins (kinesins and dyneins) using the energy of ATP hydrolysis to move vesicles along microtubule tracks. Agents interfering with microtubule trafficking include, colchicine which binds to tubulin, nocodazole which is a rapid action inhibitor, and taxol which binds to the tubulin polymer to stabilize microtubules. (ii) Another cytoskeletal network important for vesicle dynamics, particularly at the plasma membrane during fission and fusion, is the microfilament network that comprises actin fibres associated with many actin binding proteins, e.g., myosin, tropomyosin, α -actinin, and fimbrin. Agents that interfere with the functioning of the actin cytoskeleton include cytochalasins which bind to F-actin and inhibit

polymerization, latrunculin which binds to G-actin and prevents polymerization and formation of F-actin, and phallotoxins (e.g., phalloidin) which stabilizes actin filaments against a variety of depolymerizing or even denaturing stimuli.

The reader is referred to the following articles on endocytosis: Stamnes (200);⁴¹ Mousavi et al. (2004);⁴² Maxfield and McGraw (2004);⁴³ Smith and Helenius (2004);⁴⁴ and Bishop (2003).⁴⁵

7.6 NUCLEASES AND PEPTIDASES

Chemical degradation is critical biological barrier hindering the successful delivery of polynucleotide and peptide or protein-based drugs, and as such some mention of nucleases and peptidases within this chapter is warranted.

7.6.1 NUCLEASES

Nucleases are a class of enzymes that catalyze the hydrolysis of nucleic acids, either DNA or RNA. Based on the substrate type, a nuclease is either an RNase and DNase enzyme, with a RNase catalyzing the hydrolysis of RNA and a DNase hydrolyzing DNA. Nucleases are phosphoidesterases with a tremendous variability in their substrate requirements. They are classified by their specificity as either exonucleases, which require a free terminal to bring about hydrolysis, or as an endonuclease which means the enzyme can bring about hydrolysis at any internucleotide bond even when no free termini are available as for example in a covalently closed circular DNA. Some nucleases have both, endo- and exo-activities. Some are specialized for single-stranded DNA (ss-DNA) others for double-stranded DNA (ds-DNA); again, some nucleases can work on both types. Further, some exonucleases work in a $3' \rightarrow 5'$ direction, others in a $5' \rightarrow 3'$ direction. Some endonucleases comprise site-specificity and require distinct sequences for cleavage others require helper proteins to find their targets. Many key processes within cells are controlled through nucleases, for example, protective mechanisms against invasion by microbial DNA or RNA, degradation of host cell DNA after virus infections, DNA repair, DNA recombination, DNA synthesis, DNA packaging in chromosomes and viral compartments, and maturation of RNAs or RNA splicing. Nucleases will clearly also degrade therapeutically administered DNA or RNA species if these are not given sufficient protection from the pharmaceutical delivery systems.

DNase I as an endonuclease attacks DNA at points within the nucleic acid polymer. In mammalian cells, the site for the catalytic activity of DNase I comprises a five amino acid residue including: Q-39, Q-78, H-136, D-212, and H-252. DNase I gene (3.2 kb) comprises nine exons separated by eight introns. The DNase I isoforms that exist across mammalian species possesses a number of common properties such as molecular mass of 30–40 kDa, and optimal working pH of 6.5 to 8.0, activation by the divalent cations Mg²⁺ and Ca²⁺, and inhibition by the divalent chelators EDTA or EGTA.
DNase II enzymes belong to a family of highly homologous DNases which act primarily in engulfment-mediated DNA degradation. DNase II exhibits an acidic pH optimum for activity of approximately pH 5.0. Thus pH and sequestration within the lysosomal compartment are the primary means by which DNase II activity is regulated *in vivo*. DNase II enzymes hydrolyze the phosphodiester backbone of DNA molecules by a single strand nicking mechanism which generates 3'-phosphate groups. Biochemically, this cleavage event is mediated by a catalytic centre that contains a critical histidine residue and does not depend upon the presence of divalent cations. DNase II activity can be inhibited by certain divalent cations such as zinc and copper. In addition, cations such as sodium also have inhibitory effects at increasing concentrations. Cleavage-site preferences of DNAse II enzymes can be most readily detected during the middle phase of DNA catabolism. It is believed that DNase II interacts with at least three DNA base pairs during the cleavage process. Human DNase II represents a major band at approximately 35 kDa.

7.6.2 PEPTIDASES

Peptidases define enzymes that mediate peptide bond hydrolysis, and the term protease is considered synonymous with that of peptidase. Peptidases are involved in a very wide range of physiological processes, performing either (i) limited hydrolysis in which only one or a limited number of peptide bonds within a target protein are cleaved, and leading to the activation or maturation of a formerly inactive protein, or (ii) unlimited hydrolysis in which peptides or polypepides are degraded into their amino acid constituents. The susbtrates to be degraded are usually first conjugated to ubiquitin which marks them for rapid hydrolysis within the proteasome. Another pathway for unlimited proteolysis consists of peptide bond digestion within late endosomes and lysosomes.

Peptidases comprise two groups of enzymes: endopeptidases which cleave peptide bonds at points within a polypeptide, and exopeptidases, which remove amino acids sequentially from either N- or C-terminus. The term proteinase is formerly used as a synonym for endopeptidases, and four mechanistic classes of proteinases exist including: serine proteinases; cysteine proteinases; aspartic proteinases, and metallo-proteinases. The serine proteinases comprise: (a) the chymotrypsin family which includes the mammalian enzymes such as chymotrypsin, trypsin or elastase or kallikrein and, (b) the substilisin family which include the bacterial enzymes such as subtilisin. The active site in each serine protease includes a serine residue, a histidine residue, and an aspartate residue. During catalysis, there is nucleophilic attack of the hydroxyl oxygen of a serine residue of the protease on the carbonyl carbon of the peptide bond that is to be cleaved. An acyl-enzyme intermediate is transiently formed. The cysteine proteinases comprise plant proteases such as papain, actinidin, or bromelain and mammalian lysosomal proteases such as the cathepsins, and cytosolic calpains (calcium-activated). Catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. The aspartic proteinases comprise in the main enzymes belonging to the pepsin

family, which includes digestive enzymes such as pepsin and chymosin as well as lysosomal cathepsins D and processing enzymes such as renin, and certain fungal proteases (penicillopepsin, rhizopuspepsin, and endothiapepsin). A second aspartic proteinase family comprises viral proteinases such as the protease from the AIDS virus (HIV) also called retropepsin. The pairing of two aspartate residues within the catalytic site is critical. In contrast to serine and cysteine proteases, catalysis by aspartic proteinases do not involve in the formation of a covalent intermediate. The metallo proteinases comprise a wide range of enzyme families differing widely in their sequences and their structures but the majority contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel. Metallo proteinases include the digestive carboxypeptidase enzymes, and various matrix metalloproteases, e.g., collagenase, that are involved in degradation of the extracellular matrix during tissue remodeling.

The cysteine proteinases, in particular cathepsins B, H, and L, together with the aspartate proteinase, cathepsin D, appear to be the most active intravesicular proteases. Cathepsin B exists in the plasma membrane and early endosomes, and cleaves after arginine residues in short peptide substrates, and also exerts a nonspecific carboxy-terminal dipeptidyl peptidase activity. Cathepsin H possesses endopeptidase activity smilar to that of cathepsin B on short peptide substrates. In addition it displays aminopeptidase activity. Cathepsin L displays endopeptidase activity cleaving after hydrophobic residues. Cathepsin D, present in macrophage endosomes, cleaves after aromatic- and also long-side-chain containing amino acids. For a review see Brockman and Murphy (1993).⁴⁶

Exopeptidases are enzymes which cleave di- and tripeptides into their constituent amino acids. They differ from endopeptidases in that they target terminal peptide bonds. Exopeptidases can be divided into aminopeptidases, dipeptidases, dipeptidyl peptidases and tripeptidyl peptidases, peptidyldipeptidases, carboxypeptidases, and omega peptidases. Aminopeptidases are a subclass of exopeptidases that act on the free N terminus end of a polypeptide liberating a single amino acid residue. Dipeptidases are exopeptidases that specifically act on dipeptides. Dipeptidyl peptidases cleave dipeptides from the amino terminal of a polypeptide. Dipeptidyl peptidase I (also called cathepsin C) is an expopeptidase which removes N-terminal dipeptide esters or amides at the acidic pH found within lysosomes. Dipeptidyl peptidase II shows activity only against tripeptides cleaving at prolyl bonds. Dipeptidyl peptidase III catalyses release of an N-terminal dipeptide from a peptide comprising four or more residues, with broad specificity. Dipeptidyl peptidase IV catalyzes the release of an N-terminal dipeptide at the carbonyl side of a proline residue. Peptidyl-dipeptidases catalyze the release of a C-terminal dipeptide, generally membrane-bound and active at neutral pH, e.g., angiotenson-converting enzyme. Carboxyesterases are enzymes that act at a free C-terminus of a polypeptide to liberate a single amino acid residue. They are further divided based on their catalytic mechanism into serine-type carboxypeptidases, metallocarboxypeptidases, and cysteine-type carboxypeptidases.

7.7 EPITHELIAL AND ENDOTHELIAL TISSUE BARRIERS

7.7.1 GASTROINTESTINAL

The epithelium of the stomach is principally concerned with secretion. The pH of gastric juice is normally pH 1–2 on a fasting stomach, increasing up to pH 4 following the ingestion of food. The small intestine comprises the distinct zones of duodenum, jejunum, and ileum. The first part of the small intestine, the duodenum, functions to neutralize the gastric acid and initiate further digestive processes. Pancreatic secretions and bile duct secretions enter at the level of the duodenum. Food absorption occurs mainly in the jejunum and ileum. The epithelial cell lining the intestinal mucosa is the enterocyte, which is a columnar epithelial cell forming a monolayer epithelium. The pH of the small intestine increases from about 5.5 in the duodenum to pH 6–7 in the jejunum and ileum. The luminal foldings of the small intestinal mucosa and submucosa, the villi which bear at their apical surface the enterocytes, and the microvilli present on the surface of each enterocyte itself, increase the surface area potentially available for drug absorption across the small intestine to approximately 200 m^2 .

The rate and extent of drug absorption from the small intestine depends on the release of the active ingredient from a dosage form, its solubility in the liquid phase of gastrointestinal contents, and the transport of the dissolved compound or the intact dosage form from the stomach into the duodenum. In dynamic systems such as dosing to the gastro-intestinal tract, drug absorption is dependent on the residence time of the drug in solution at an absorption site. As such gastric emptying and intestinal motility can be critical determinants of drug absorption.

The oral route of administration for protein and nucleic acid drugs is quite problematic because in addition to proteolysis in the stomach, the high acidity of the stomach destroys them before they can reach the intestine for absorption. Polypeptides and protein fragments, produced by the action of gastric and pancreatic enzymes are further cleaved by exo- and endopeptidases in the intestinal brush border membrane to yield di- and tripeptides, and even if proteolysis by pancreatic enzymes is avoided, polypeptides are subject to degradation by brush border peptidases. Any of the peptides that survive passage through the stomach are further subject to metabolism in the intestinal mucosa, where penetration barrier prevents entry into cells.

7.7.2 ORAL

Absorption from the oral cavity includes: (i) sublingual drug delivery across the epithelial lining of the ventral surface of the tongue and of a region of the mouth itself underlying the tongue, and (ii) buccal drug delivery across the epithelial lining of the cheeks. Both sublingual and buccal regions of the oral cavity pertinent to drug delivery comprise stratified squamous nonkeratinized epithelium, with a thickness of the sublingual mucosal barrier $\sim 200 \,\mu\text{m}$ and a thickness of the buccal mucosal barrier of 500–800 μm . Both regions receive a

relatively good blood supply, with molecules absorbed from these sites not subject to first-pass metabolism of the gut mucosa and liver. The buccal route of delivery allows the opportunity for application of adhesive drug delivery systems able to release drug over a prolonged period of time, and this is not applicable to the sublingual route where system retention and flow of saliva makes such systems less amenable.

7.7.3 LUNG

For the treatment of local lung disease such as emphysema, asthma, chronic obstructive pulmonary disease, cystic fibrosis, and chronic bronchitis, aerosol administration of drugs can be undertaken at much lower doses than those used for the equivalent oral or intravascular dosing of these agents. Proteins and peptides appear to show good bioavailability across the epithelium of the deep lung compared to other barriers such as in the gastrointestinal tract or across skin. For example, insulin absorption for via inhalation drug delivery approximates 10–20%. The predominant mechanism of absorption for proteins from lung airspace to blood is still unclear although a range of processes from passive paracellular diffusion to active vesicular trafficking have been considered.

The respiratory system itself comprises two distinct regions, the upper and lower respiratory tracts. The upper respiratory tract consists of the nasal and paranasal passages, and the pharynx (collectively termed the nasopharyngeal region). The nasopharyngeal region serves to warm and humidify inhaled air and prevents the passage of particulates and micro-organisms to the membranes of the lower respiratory tract. The trachea is the first conducting airway and connects the nasopharyngeal region to the lower respiratory tract lung tissue. The main function of the conducting airways is to carry inspired air through to the gaseous exchange region of the alveoli. The conducting airways are a series of bifurcations, the trachea bifurcates into the bronchi which subsequently bifurcate into bronchioles, etc. After about 12 generations of airway bifurcation cartilage disappears from the airway walls and the airways (now termed bronchioles) rely on lung volume to maintain airway caliber. The epithelium of the proximal bronchi are lined with pseudo-stratified ciliated columnar epithelium, with the epithelial cells (cell depth \sim 50 µm) interspersed with secretory and basal cells. More distal within the conducting airways the epithelium becomes progressively cuboidal (cell depth $\sim 10 \,\mu\text{m}$), and with the ciliated cells becoming more sparse in number. Approximately 40% of the epithelial cells lining the conducting airways of mammals are ciliated. These cilia beat in one direction only, propelling the airway surface liquid upward toward the pharyngeal region. The airway surface liquid comprises an aqueous sol phase which lies proximal to the surface epithelium surrounding the cilia, and a higher viscosity gel phase which sits on top of the cilia. The principal macromolecular structural component of the gel phase is mucin glycoproteins, secreted from goblet cells of the surface epithelium and serous cells of the submucosal tissue. It is the cilia driven movement of this gel upward toward

the pharyngeal region which constitutes the mucociliary clearance mechanism of the lung, the function of which is to clear deposited particulate matter trapped within the mucus barrier.

After about 16 generations of airway bifurcation the conducting airway to respiratory transitional zone begins which eventually gives rise to the alveolar sacs. Each alveolus (diameter 250 µm) is lined by alveolar epithelium and supported by a thin basement membrane which interfaces with numerous blood capillaries in such an arrangement that presents an extensive air to blood network with separation by only a minimal tissue barrier and therefore allowing for optimal diffusion of gases. The alveolar epithelium is comprized up to 93% of its surface area by the squamous alveolar epithelial type I cell which is extremely thin with an average cell thickness of 0.35 µm ranging from $2-3\,\mu\text{m}$ in the perinuclear region of the cell to approximately $0.2\,\mu\text{m}$ in the peripheral attenuated regions of the cell. The total alveolar epithelial surface area within an average adult human lung has been estimated, using electron microscopy techniques, to be as large as 140 m^2 ; this contrasts with an approximate 2 to 4 m^2 surface area for the tracheo-bronchiole conducting airways. The alveolar epithelial surface is covered with a surface film of surfactant that lowers the surface tension in lungs and is essential if the alveolar sacs are to expand during inspiration. The volume of this alveolar film has been calculated to be 7–20 ml per 100 m^2 alveolar surface area.

Macrophages are also present in the alveolus and are able to migrate across the alveolar epithelium; they are found at all the lung epithelial surfaces. Since there are no cilia in the alveoli, the macrophages are the first line of defence and engulf exogenous and endogenous particles that have escaped from the mucous trap and the ciliary escalator of the upper respiratory tract.

Some of the above morphometric features of the alveolar epithelial– pulmonary capillary barrier (e.g., large surface area, thin cellular barrier, absorptive surface beyond the mucociliary escalator, and high tissue blood perfusion) exemplify the "favorable" anatomical determinants to be considered in delivering proteins and peptides to the systemic circulation and have driven the challenge to improve the delivery of therapeutic aerosols to the lung periphery, and in particular the alveolar epithelium.

The therapeutic benefit from a medical aerosol containing drug depends upon the airway geometry, humidity, clearance mechanism, and size of the aerosol particles depositing on the lung epithelial surface. The aerodynamic behavior of aerosol particles within the respiratory tract is described by the distinct processes of inertial impaction, sedimentation, and diffusion. Significant device and aerosol technology has been committed to obtaining optimal deposition patterns for therapeutic aerosols.

7.7.4 NASAL

Drugs may be delivered to the nasal cavity for local action, such as nasal decongestants, or for the purposes of achieving systemic drug absorption.

Examples of prescription available drugs delivered nasally for the latter objective include not only low molecular weight organic, molecules but also peptides such as buserelin, desmopressin, oxytocin, and calcitonin. The physicochemical properties of a molecule which affect its absorption across the nasal epithelium are broadly the same as those affecting transepithelial absorption at any site, although the rate of degradative drug metabolism within the nasal cavity and mucosa is certainly less than that seen in the gastrointestinal tract; absorption via the nose avoids hepatic first pass metabolism.

The nasal cavity itself extends from the nostrils to the nasopharynx and is divided laterally by the nasal septum. The total surface area of the nasal cavity is about 150 cm². The nostrils lead into the nasal vestibule, the most proximal zone of the nasal cavity, which is lined by squamous epithelium. From the vesitibule the cavity extends toward the turbinates (inferior, middle, superior) which constitute the main part of the nasal passage. The turbinates are delicate spiral bones found in the nasal passages covered with highly vascularized pseudostratified ciliated columnar secretory epithelium. Microvilli are found on the columnar cells which increases the surface area available for absorption of drugs. The main function of the turbinates is in humidification/ dehumidification of inspired/expired air. The olfactory region of the nose is located at the roof of the nasal cavity close to the superior turbinate and is lined with nonciliated neuro-epithelium.

The ciliated epithelium overlying the turbinates takes part in the mucociliary clearance of particulate matter, such as dust and microorganisms, that deposit upon the mucus blanket lining this region of the nasal cavity. The mucus with entrapped particulates is propelled by the cilia towards the nasopharynx to be either swallowed or expectorated. The clearance of the bulk of the mucus from the nose to the nasopharynx occurs over 10–20 min.

Drugs are administered to the nasal cavity as aqueous drops, or aerosol sprays. Nasal drops disperse a drug solution throughout the length of the nasal cavity from the vestibule to the nasopharynx, offering a relatively large area for immediate absorption. Nasal sprays produce an aerosol of drug containing droplets analogous to the aerosols produced in pulmonary drug delivery, although the device technology is of a much simpler design. Aerosol droplets produced by nasal sprays tend to deposit drug at the front of the nasal cavity with little reaching the turbinates. The significance upon absorption of the above deposition patterns is that nasal drops while instantly spread throughout the full nasal cavity will also be subject to the immediate effects of mucociliary clearance, thus limiting the residence time drug remains in contact with the absorption surface. In contrast, aerosols while depositing initially in the nonciliated regions of the anterior nasal passage will have a much longer residence time at the absorption surface, although with time an increasing proportion of the drug aerosol will be subject to clearance by mucociliary transport.

A range of strategies have been explored to increase the absorption of drugs from the nasal cavity including the use of penetration enhancers to alter nasal epithelium permeability, and the use of bioadhesives to improve contact, and prolong residence time, with the nasal mucosa. There is increasing evidence that drugs administered to the roof of the nasal cavity proximal to the olfactory region may gain direct access to the central nervous system, without having to cross the blood-brain barrier.

7.7.5 Skin

The skin is an extremely efficient barrier that minimizes water loss from the body. As such drug absorption across the skin is potentially more limiting and the relationship between a drug's physicochemical properties and epidermal permeability are less ambiguous than with mucosal barriers. The body's skin surface area is approximately 1.5 m^2 and respresents a readily accessible surface for application of drug delivery systems. The outermost region of the skin is the epidermis (100-250 µm thickness) which is a stratified, squamous, keratinizing epithelium with keratinocytes constituting the major cellular component (>90% of the cells). Underlying the epidermis is the dermis which comprises primarily connective tissue and provides support to the epidermis. It contains blood and lymphatic vessels and nerve endings. The dermis also bears the skin's appendageal structures, specifically the hair follicles and sweat glands. The epidermis is avascular and for drugs to gain access to the capillary network they must traverse the full thickness epidermis to reach the underlying vascularized dermis. Drugs absorbed across the skin avoid hepatic first-pass metabolism.

The epidermis is divided histologically into five distinct layers corresponding to the sequential nature of keratinocyte cell differentiation from the basal layer, stratum basale, which bears keratinocyte stem cells and is the site for proliferation of new keratinocytes, to the outermost layer, stratum corneum, bearing terminally differentiated keratinocytes; keratinocyte differentiation and migration from stratum basale to stratum corneum is a continuous process taking 20–30 days in duration. However, it is the stratum corneum comprising approximately 20 cell layers in depth, that provides the principal barrier to skin. An often used analogy for the stratum corneum is that of a "brick wall" with the fully-differentiated stratum corneum keratinocytes, or corneocytes as they are alternatively known, comprising the "bricks," embedded in a "mortar" constituted by intercellular lipids which include ceramides, cholesterol, and free fatty acids. An obvious distinction should now be apparent in that the intercellular (paracellular) pathway in mucosal barriers is aqueous in nature, while the intercellular pathway in the stratum corneum barrier is lipid in nature. It is the convoluted lipid intercellular pathway that is considered the primary route for drug permeability across the skin barrier.

From the above it should be clear that lipophilicity is a key physicochemical drug property for stratum corneum permeability with optimum log[octanol-water] partition coefficients for transport in the range of 1–3. For very lipophilic compounds, e.g., log[octanol-water] partition coefficients >4, however, then the rate-limiting step in absorption may indeed be the

partitioning of the drug from the stratum corneum into the underlying more aqueous viable epidermis. The potential with this kind of molecule is for significant lag times in absorption and for the stratum corneum serving as a reservoir for the drug even after removal of the delivery system. Partitioning of charged or very polar molecules into the stratum corneum is essentially nonexistent without the use of some form of pentration enhancement.

Chemical penetration enhancers will work by either: (i) facilitating the partitioning of drug from the vehicle into the epidermis; (ii) reducing the diffusional barrier of the stratum corneum by perturbing the intercellular lipid pathway, or promoting transport via the appendages. Iontophoresis is an interesting form of penetration enhancement facilitating the delivery of charged drug species by the application of an applied electrical potential.

7.7.6 CAPILLARY TRANSFER

A number of factors influence the cellular transport of substances from the blood plasma to organ through the vasculature endothelium, among which physiochemical properties of the soluble substances in plasma (e.g., nutrients and administered drugs), vascular driving forces (i.e., hydrostatic and oncotic pressure gradients), and also the endothelium membrane characteristics such as paracellular junctions and carrier- and/or receptor-mediated transport systems. Transport to tissues also depends to a significant extent on the physiochemical characteristics of the components underlying the endothelium (i.e., basement membrane, extracellular matrix, interstitial fluid).

Mechanisms of microvascular permeability of macromolecules as large as fibrinogen between the blood and the tissues have been widely investigated. It has been shown that such transport can be linked closely with net fluid filtration, i.e., macromolecules were carried through microvascular walls by convection via large pores. However, the transytosis of macromolecules via coated or noncoated vesicles of membrane domains (rafts and/or caveolae) also have been reported, particularly in those microvascular beds where the endothelium is continuous. Brain capillary endothelial cells generate tight junctional barriers which limit macromolecules transport across this barrier to vesicular trancytosis.

7.8 CONCLUDING REMARKS

Biology is now an intrinsic element underpinning pharmaceutical sciences. Innovative developments in drug delivery will require integration of engineering, physical-chemical and biological sciences. This chapter has provided only a brief overview of some of the biology pertinent to the drug delivery of polynucleotide and polypeptide drugs. Each element represents an extensive research field in its own right and the reader will be required to draw upon much more of the literature before he or she can effectively incorporate this into their own scientific ventures.

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8 Pharmacokinetics of Protein- and Nucleotide-Based Drugs

Bernd Meibohm

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8.1 INTRODUCTION

In the last two decades, protein therapeutics have grown from largely niche products to mainstream pharmacotherapeutic agents. Recent business development analyses of the pharmaceutical industry suggest that protein drugs will even have a substantially larger share in the pharmaceutical markets of the future. Antibody drugs alone are projected to constitute half of all new active substances to be developed within the next 10–15 years.¹

The basis for the pharmacotherapeutic use of proteins is similar to that of small molecules, a defined relationship between the intensity of the therapeutic effect and the amount of drug in the body, or, more specific, the drug concentration at its site of action, i.e., a defined exposure-response relationship. Systemic exposure can be described either in cumulative terms (for example area-under-the-concentration-time-curve, AUC) or as concentration-time profiles in biological fluids such as plasma or blood or in the target tissue. The relationship between exposure and response may be either simple or complex, and thus obvious or hidden.² However, if no simple relationship exists at all rather than that it is not readily apparent.³ Thus, systemic exposure is a crucial factor in the successful development of highly efficacious protein therapeutics by guiding the preclinical and clinical drug development process as well as drug delivery strategies.⁴

Protein drugs are generally subjected to the same pharmacokinetic principles as traditional, small molecule drugs. Based on their similarity to endogenous compounds or nutrients, however, numerous caveats and pitfalls related to pharmacokinetics and systemic exposure have to be considered during the development process that may require additional resources. Especially drug delivery has been a major focus in protein drug development due to the generally low bioavailability of protein therapeutics if administered via noninvasive administration techniques, e.g., via the oral or transdermal route. Drug targeting to facilitate preferential uptake of protein drugs into the organs and cells that constitute their primary target tissue have also achieved special attention. These topics, however, are extensively discussed in other chapters of this book. Thus, this chapter will primarily focus not on drug delivery and absorption of protein drugs, but on the disposition and elimination characteristics of protein-based therapeutics. These are applicable independent of the route of drug administration, and constitute besides bioavailability the major factors that govern the systemic exposure to these agents. For a more general discussion of pharmacokinetic principles, the reader is referred to several textbooks and articles that review the topic in extensive detail.5-10

Nucleotide-based drugs as a novel pharmacotherapeutic approach face similar challenges as protein drugs with regard to drug delivery as well as drug disposition since nucleotide-based drugs are also macromolecules with structures similar to those of endogenous substances. Hence, there will also be a section in this chapter devoted to the pharmacokinetics of oligonucleotides and plasmid DNA.

8.2 PHARMACOKINETICS OF PROTEINS

The *in vivo* disposition of peptide and protein drugs may often be predicted to a large degree from their physiological function. Peptides, for example, which frequently have hormone activity, usually have short elimination half-lives, which is desirable for a close regulation of their endogenous levels and thus

function. Insulin, for example shows dose-dependent elimination with a relatively short half-life of 26 and 52 min at 0.1 and 0.2 U/kg, respectively. Contrary to that, proteins that have transport tasks such as albumin or long-term immunity functions such as antibodies have elimination half-lives of several days, which enables and ensures the continuous maintenance of necessary concentrations in the blood stream.¹¹ This is for example reflected by the elimination half-life of antibody drugs like the anti-epidermal growth factor receptor antibody cetuximab, for which a half-life of approximately 7 days was reported.¹²

8.2.1 **PROTEIN ABSORPTION**

Although oral drug delivery is for the majority of pharmacotherapeutics the most preferable route of administration due to its convenience, costeffectiveness, and painlessness, most protein drugs are currently formulated as parenteral formulations because of their poor oral bioavailability. The lack of oral bioavailability is mainly caused by two factors, high gastrointestinal enzyme activity and the function of the gastrointestinal mucosa as absorption barrier. The gastrointestinal tract is the most efficient body compartment for peptide and protein metabolism due to substantial peptidase and protease activity. In addition, the gastrointestinal mucosa presents a major absorption barrier for water-soluble macromolecules such as peptides and proteins.^{13–15} Thus, although various factors such as permeability, stability and gastrointestinal transit time can affect the rate and extent of orally administered proteins, molecular size is generally considered the ultimate obstacle.¹⁶

Since oral administration is still a highly desirable route of drug delivery for protein drugs, several promising strategies have recently emerged from intensive research into methods to overcome the obstacles associated with oral drug delivery of proteins. Approaches to increase the oral bioavailability of protein drugs include absorption enhancers for increasing the amount of drug that is able to cross absorption barriers as well as encapsulation into microor nanoparticles thereby protecting proteins from intestinal degradation.^{16–18} Other strategies include chemical modifications such as amino acid backbone modifications and chemical conjugation to improve the resistance to degradation and permeability of protein drugs. Coadministration of protease inhibitors has also been suggested for the inhibition of enzymatic degradation.^{17,19}

The lack of systemic activity after oral administration for most proteins resulted besides the frequently used, but invasive intravenous (IV) application into the utilization of numerous non-oral administration pathways, e.g., nasal, buccal, rectal, vaginal, percutaneous, ocular, or pulmonary drug deliveries.²⁰ In addition, drug delivery by subcutaneous (SC) or by intramuscular (IM) administration are frequently used alternatives for administering protein drugs. Presystemic degradation processes, however, are also frequently associated with these administration routes, resulting for example in a reduced bio-availability of numerous proteins after SC or IM administration compared to their IV administration. The pharmacokinetically derived apparent absorption

rate constant for protein drugs administered via these administration routes is thus the combination of absorption into the systemic circulation and presystemic degradation at the absorption site. The true absorption rate constant k_a can then be calculated as

$$k_{\rm a} = F \cdot k_{\rm app}$$

where *F* is the bioavailability compared to intravenous administration. A rapid apparent absorption rate constant k_{app} can thus be the result of a slow absorption and a fast presystemic degradation, i.e., a low systemic bioavailability.¹³

Other potential factors that may limit bioavailability of proteins after SC or IM administration including variable local blood flow, injection trauma, and limitations of uptake into the systemic circulation related to effective capillary pore size and diffusion.

After subcutaneous administration, large molecule drugs like proteins may, dependent on their molecular weight, either enter the systemic circulation via blood capillaries or through lymphatic vessels, as illustrated in Figure 8.1.²¹ Macromolecules larger than 16 kD are predominantly absorbed into the lymphatics whereas those under 1 kD are mostly absorbed into the blood circulation.²² This is of particular importance for those agents whose therapeutic targets are lymphoid cells, for example interferons and interleukins.



FIGURE 8.1 Relationship between lymphatic uptake and molecular weight of drugs administered subcutaneously. The graphic shows the correlation between molecular weight and the cumulative recovery (mean \pm SD) of rINF α -2a (MW 19,000), cytochrome c (MW 12,300), inulin (MW 5200) and 5-fluoro-2'-deoxyuridine (FUDR) (MW 246) in the efferent lymph from the right popliteal lymph node following SC administration into the lower part of the right hind leg in sheep (n=3). The linear regression line has a correlation coefficient r of 0.998 (p < 0.01). (From Supersaxo, A., Hein, W. R., and Steffen, H., *Pharm. Res.*, 1990; **7:** 167, with permission.)

SC administration of recombinant human interferon α -2, for example, resulted in high interferon α -2 concentrations in the lymphatic system, which drains into the regional lymph nodes.²³

8.2.2 PROTEIN DISTRIBUTION

The distribution volume of proteins is determined largely by their molecular weight, physiochemical properties (e.g., charge, lipophilicity), protein binding, and their dependency on active transport processes. Since most therapeutic proteins have high molecular weights and are thus large in size, their apparent volume of distribution is usually small and limited to the volume of the plasma or the extracellular space, predominantly because of their limited mobility secondary to impaired passage through biomembranes.²⁴ Active tissue uptake and binding to intra- and extravascular proteins, however, can substantially increase the volume of distribution of protein drugs, as reflected by the relatively large volume of distribution of up to 2.8 L/kg for interferon β -1b.²⁵

The plasma concentration-time profiles for proteins after IV administration are usually biphasic and can best be described by a two-compartment pharmacokinetic model.¹³ A biexponential concentration-time profile has, for example, been described for clenoliximab, a macaque-human chimeric monoclonal antibody specific to the CD4 molecule on the surface of T-lymphocytes.²⁶ Similarly, AJW200, a humanized monoclonal antibody to the von Willebrand factor, exhibited biphasic pharmacokinetics after IV administration.²⁷ The central compartment in this two-compartment model represents primarily the vascular space and the interstitial space of well-perfused organs with permeable capillary walls, including the liver and the kidneys. The peripheral compartment is more reflective of concentration-time profiles in the interstitial space of poorly-perfused tissues like skin and inactive muscle.

The central compartment in which peptides and proteins initially distribute after intravenous administration has thus typically a volume of distribution equal or slightly larger than the plasma volume, i.e., 3-8 L. The total volume of distribution frequently comprises with 14–20 L not more than twice the initial volume of distribution.^{13,27} An example for such a distribution pattern is the tissue plasminogen activator (t-PA) analogue tenecteplase. Radiolabeled ¹²⁵I-tenecteplase was described to have an initial volume of distribution of 4.2–6.3 L and a total volume of distribution of 6.1–9.9 L with liver as the only organ that had a significant uptake of radioactivity. The authors concluded that the small volume of distribution suggests primarily intravascular distribution for tenecteplase, consistent with the drug's large molecular weight of 65 kD.²⁸

There is a tendency for the initial and the total volume of distribution to correlate with each other, which implies that the volume of distribution is predominantly determined by distribution into the vascular and interstitial space as well as unspecific protein binding in these distribution spaces. The distribution rate is inversely correlated with molecular size and is similar to that of inert polysaccharides, suggesting that passive diffusion through aqueous channels is the primary distribution mechanism.¹⁴

Another factor that can influence the distribution of proteins is binding to other endogenous proteins. Physiologically active, endogenous proteins frequently interact with specific binding proteins involved in their transport and regulation. Furthermore, interaction with binding proteins may enable or facilitate cellular uptake processes and thus affect the drug's pharmacodynamics. Similarly, therapeutically administered protein drugs may interact with endogenous binding proteins.

It is a general pharmacokinetic principle, which is also applicable to proteins, that only the free, unbound fraction of a drug substance is accessible to distribution and elimination processes as well as interactions with its target structure at the site of action, for example a receptor or ion channel. Thus, protein binding may affect the pharmacodynamics, but also disposition properties of protein therapeutics. Specific binding proteins have been identified for numerous protein drugs, including recombinant human DNases for use as mucolytics in cystic fibrosis,²⁹ growth hormone,³⁰ and recombinant human vascular endothelial growth factor (rhVEGF).³¹

Protein binding not only affects the unbound fraction of a protein drug and thus the fraction of a drug available to exert pharmacological activity, but many times it also may either prolong protein circulation time by acting as a storage depot or it may enhance protein clearance. Recombinant cytokines, for example, may after IV administration encounter various cytokine-binding proteins including soluble cytokine receptors and anti-cytokine antibodies.³² SC administration of glycosylated recombinant human interleukin-6 (rhIL-6) evaluated as a thrombopoetic agent in patients with advanced cancer resulted in an apparent attenuation in circulating IL-6 concentrations with continuing injections, which seems to be related to soluble interleukin 6 receptor concentrations.³³ The enhancement of IL-6 clearance via administration of cocktails of three anti-IL-6 monoclonal antibodies was suggested as therapeutic approach in cytokine-dependent diseases like multiple myeloma, B-cell lymphoma, and rheumatoid arthritis.³⁴ The authors could show that, while the binding of one or two antibodies to the cytokine led to stabilization of the cytokine, simultaneous binding of three anti-IL-6 antibodies to three distinct epitopes induced rapid uptake of the complex by the liver and thus mediated a rapid elimination of IL-6 from the central compartment.

Aside from physicochemical properties and protein binding of proteinbased drugs, site-specific and target-oriented receptor mediated uptake can also influence biodistribution. Therefore, there is often a close interrelationship between distribution, elimination, and pharmacodynamics for protein therapeutics in contrast to conventional small molecule drugs. The generally low volume of distribution of protein drugs should not necessarily be interpreted as low tissue penetration. Receptor-mediated specific uptake into the target organ, as one mechanism, can result in therapeutically effective tissue concentrations despite a relatively small volume of distribution.

8.2.3 PROTEIN ELIMINATION

Protein-based therapeutics are generally subject to the same metabolic pathways as endogenous or dietetic proteins. The end products of protein drug metabolism are thus amino acids that are reutilized in the endogenous amino acid pool for the de novo biosynthesis of structural or functional proteins in the human body. Detailed investigations on the metabolism of proteins are relatively difficult because of the myriad of potential molecule fragments that may be formed, and are therefore generally not conducted. Nonmetabolic elimination pathways such as renal or biliary excretion are negligible for most proteins. If biliary excretion occurs, however, it is generally followed by subsequent metabolic degradation of the compound in the gastrointestinal tract (see Section 8.2.3.2).¹³

The metabolic rate for protein degradation generally increases with decreasing molecular weight from large to small proteins to peptides, but is also dependent on other factors like secondary and tertiary structure as well as glycosilation. The clearance of a peptide or protein describes the irreversible removal of active substance from the intracellular space, which includes besides metabolism also cellular uptake. Proteolytic degradation of proteins can occur unspecifically nearly everywhere in the body or can be limited to a specific organ or tissue. Due to this unspecific proteolysis of some proteins already in blood as well as potential active cellular uptake, the clearance of protein drugs can exceed cardiac output, i.e., > 5 L/min for blood clearance and > 3 L/min for plasma clearance.¹³

8.2.3.1 Proteolysis

Protein metabolism occurs especially in liver, kidneys, and gastrointestinal tissue. Proteolytic enzymes, however, are not limited to classical drug elimination organs, but are also present in blood and other body tissues, predominantly localized on cell membranes as well as within cells, for example in lysosomes. Thus, intracellular uptake is per se more an elimination rather than a distribution process.¹³

Molecular weight determines the major metabolism site as well as the predominant degradation process.³⁵ While proteolytic enzymes like peptidases and proteases in the gastrointestinal tract and in lysosomes are relatively unspecific, soluble peptidases in the interstitial space and exopeptidases on the cell surface have a higher selectivity and determine the specific metabolism pattern of an organ.¹⁴

8.2.3.2 Gastrointestinal Protein Metabolism

As pointed out under section 8.2.1, the gastrointestinal tract is a major site of protein metabolism with high proteolytic enzyme activity due to its primary function to digest dietary proteins. Thus, gastrointestinal metabolism of protein drugs is one of the major factors limiting systemic bioavailability of orally administered protein drugs. The metabolic activity of the gastrointestinal tract, however, is not limited to orally administered proteins. Parenterally administered peptides and proteins may also be metabolized in the intestinal mucosa following intestinal secretion. At least 20% of the degradation of endogenous albumin, for example, takes place in the gastro-intestinal tract.¹³

8.2.3.3 Renal Protein Metabolism

The kidneys are a major site of protein metabolism for smaller sized proteins that undergo glomerular filtration. The size-selectivity cut-off for glomerular filtration is approximately 60 kD, although the effective molecule radius based on molecular weight and conformation is probably the limiting factor.³⁶ In addition to size-selectivity, charge-selectivity has also been observed for glomerular filtration where anionic macromolecules pass through the capillary wall less readily than neutral macromolecules, which in turn pass through less readily than cationic macromolecules.³⁷

For renal protein metabolism, glomerular filtration is the dominant, ratelimiting step as subsequent degradation processes are not saturable under physiologic conditions.^{13,38} Due to this limitation of renal elimination, the renal contribution to the overall elimination of proteins is dependent on the proteolytic activity for these proteins in other body regions. If metabolic activity for these proteins is high in other body regions, there is only minor renal contribution to total clearance, and it becomes negligible in the presence of unspecific degradation throughout the body. If the metabolic activity is low in other tissues or if distribution to the extravascular space is limited, however, the renal contribution to total clearance may approach 100%. This is for instance the case for recombinant human interleukin-10 (rhIL-10), for which clearance correlates closely with glomerular filtration rate, making dosage adjustments necessary in patients with impaired renal function.³⁹

After glomerular filtration, larger peptides and proteins are actively reabsorbed in the proximal tubules via endocytosis. This cellular uptake is followed by lysosomal degradation, and has been described as method of elimination for IL-2,⁴⁰ IL-11,⁴¹ and insulin.⁴² Smaller peptides like bradykinin or glucagon undergo intraluminal metabolism, predominantly by exopeptidases in the brush border membrane of the proximal tubules.⁴³ Small peptides and amino acids as final products of both processes are transcellularly transported back into the systemic circulation with contribution of the proton driven peptide transporters PEPT1 and PEPT2.⁴⁴ Therefore only minuscule amounts of intact protein are detectable in urine.

Besides intraluminal metabolism and tubular reabsorption with intracellular lysosomal metabolism, peritubular extraction from post glomerular capillaries with subsequent intracellular metabolism is an additional renal elimination mechanism for proteins. Experiments using radio-iodinated growth hormone (¹²⁵I-rGH) have demonstrated that while reabsorption into endocytic vesicles at the proximal tubule is still the dominant route of disposition, a small percentage of the hormone may be extracted from the peritubular capillaries.^{45,46}

8.2.3.4 Hepatic Protein Metabolism

The liver constitutes similar to the kidney a major site of protein metabolism. Exogenous as well as endogenous proteins undergo proteolytic degradation to dipeptides and amino acids that are reused for endogenous protein synthesis. The rate of hepatic metabolism is largely dependent on specific amino acid sequences in the protein.

A prerequisite for hepatic protein metabolism is the uptake of proteins into the hepatocytes. Small peptides may cross the hepatocyte membrane via passive diffusion if they have sufficient hydrophobicity. Uptake of larger peptides and proteins is facilitated via either various carrier-mediated, energydependent transport processes or receptor-mediated endocytosis. Hepatic uptake and clearance of tissue plasminogen activator (t-PA) as a large protein (65 kD), for example, is facilitated by mannose and asialoglycoprotein receptors and the low density lipoprotein receptor-related protein.^{47,48}

8.2.3.5 Receptor-Mediated Protein Metabolism

Numerous protein therapeutics are characterized by pharmacologic targetmediated drug disposition, which occurs when binding to the pharmacodynamic target structure affects the pharmacokinetics of a drug compound.⁴⁹ For conventional small molecule drugs, receptor binding is usually negligible compared to the total amount of drug in the body and rarely affects their pharmacokinetic profile. In contrast, a substantial fraction of a protein drug can be bound to receptors. This binding can lead to receptor-mediated uptake by endocytosis and subsequent intracellular lysosomal metabolism. Receptormediated uptake and metabolism via interaction with these generally highaffinity, low-capacity binding sites is not limited to hepatocytes. Thus, any tissue, including the therapeutic target cells, that express receptors for the drug can contribute to the protein drug's elimination.

Since the number of protein drug receptors is limited, receptor-mediated protein metabolism can usually be saturated within therapeutic concentrations, or more specifically at relatively low molar ratios between the protein drug and the receptor. As a consequence, the elimination clearance of protein drug is not constant any more, but is dose-dependent and decreases with increasing dose. Thus, receptor-mediated elimination constitutes a major source for nonlinear pharmacokinetic behavior of numerous peptide and protein drugs, i.e., a lack of dose-proportionality.

Recombinant human macrophage colony-stimulating factor (M-CSF), for example, undergoes besides linear renal elimination a nonlinear elimination pathway that follows Michaelis–Menten kinetics and is linked to a receptormediated uptake into macrophages. At low concentrations, M-CSF follows linear pharmacokinetics, while at high concentrations, nonrenal elimination



FIGURE 8.2 Nonlinear pharmacokinetics of M-CSF as an example for target-mediated drug disposition. The plot shows measured and modeled plasma concentration-time curves (mean \pm SE) after intravenous injection of 0.1 mg/kg (n = 5), 1.0 mg/kg (n = 3) and 10 mg/kg (n = 8) M-CSF in rats (From Bauer, R. J. et al., *J. Pharmacol. Exp. Ther.*, 1994; **268:** 152, with permission.)

pathways are saturated resulting in nonlinear pharmacokinetic behavior (Figure 8.2).^{50,51}

Nonlinearity in pharmacokinetics based on receptor-mediated drug disposition has also been frequently observed for monoclonal antibody drugs, for instance for the anti-HER2 humanized monoclonal antibody trastuzumab, approved for the combination treatment of HER2 protein overexpressing metastatic breast cancer. With increasing dose level, the mean half-life of trastuzumab increases and the clearance decreases, leading to overproportional increases in systemic exposure with increasing dose (Figure 8.3).⁵² Since trastuzumab is rapidly internalized after binding to its target structure on the cell surface, saturation of this elimination pathway is a likely factor for the observed dose-dependent pharmacokinetics.⁵³

8.2.4 IMMUNOGENICITY AND PROTEIN PHARMACOKINETICS

The antigenic potential of protein therapeutics may lead to antibody formation against the protein therapeutic during chronic therapy. As briefly discussed in Section 8.2.2, protein-antibody complexation can obliterate the biological activity of a protein drug, but may also modify its pharmacokinetic profile. Faster elimination of the complex occurs if the reticuloendothelial system is stimulated, slower elimination if the antibody–drug complex forms a depot for the protein drug.⁵⁴



FIGURE 8.3 Dose-dependent pharmacokinetics of trastuzumab. Serum concentrationtime profiles (mean \pm SD) of the humanized monoclonal antibody trastuzumab after first administration in patients with HER2-overexpressing metastatic breast cancer. Dose levels are 1 mg/kg (open circles), 2 mg/kg (closed circles), 4 mg/kg (open triangles), and 8 mg/kg (closed triangles). Like many other monoclonal antibodies, trastuzumab is characterized by dose-dependent pharmacokinetics, with increasing half-life and decreasing clearance if the dose level is increased. (From Tokuda, Y. et al., *Br. J. Cancer*, 1999; **81:** 1419, with permission.)

Antibody formation is of particular concern if human proteins are used in animal studies or if animal-derived proteins are applied in human clinical studies.¹¹ Most monoclonal antibodies, for example, have been derived from mice. Administration of murine antibodies to humans may result in the development of a human anti-mouse immunoglobulin antibody (HAMA) response, which is in most cases directed against the constant regions of the murine antibody. Genetically engineered mouse–human chimeric antibodies try to minimize this immunogenicity in humans by joining variable domains of the mouse to the constant regions of human immunoglobulins.⁵⁵ The anti-epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab is an example of a murine–human chimeric antibody recently approved for various cancer indications.⁵⁶

The immunogenicity of protein therapeutics is dependent on the route of administration. Extravascular injection is known to stimulate antibody formation more than IV application, which is most likely caused by the increased immunogenicity of protein aggregates and precipitates formed at the injection site.

An increasingly applied approach to reduce the immunogenicity of protein drugs is their conjugation with polyethylene glycol (PEG).^{57,58} Through steric hindrance, PEG conjugation can shield antigenic determinants on the drug from detection by the immune system.⁵⁹ The development of pegaspargase is

a successful example for overcoming a high rate of allergic reactions towards L-asparaginase using PEG conjugation techniques.⁶⁰

8.3 PHARMACOKINETICS OF OLIGONUCLEOTIDES

Antisense oligonucleotides, usually 15–25 nucleotides in length, hold great promise as novel therapeutic agents designed to specifically and selectively inhibit the production of disease-related products, with fomivirsen being the first approved antisense oligonucleotide drug product.⁶¹ Phosphorothioate oligonucleotides (PS ODNs) differ from native DNA only by substitution of a nonbridging oxygen with sulfur in the phosphodiester bridge linking the nucleotides. The sulfur substitution dramatically modifies the pharmaco-kinetics of oligonucleotides by stabilizing them against nuclease digestion and increasing nonspecific plasma protein binding, resulting in a prolonged residence time in tissues and cells, improved tissue distribution, and reduced urinary secretion.⁶² Since PS ODNs have so far advanced furthest in clinical development, with a significant body of preclinical and human pharmaco-kinetic data available, the pharmacokinetics of PS ODNs will be the focus in this section.

The pharmacokinetics of PS ODNs, including tissue distribution, metabolic degradation, and excretion, are generally sequence-independent, resulting in a shared similar pharmacokinetic profile of PS ODNs.⁶² This similarity has been observed in preclinical models as well as in humans. Figure 8.4, for example, shows the nearly superimposable plasma concentration-time profiles of four different PS ODNs in Cynomolgus monkeys, given at equivalent doses as 2-hour short-term infusions. The similarity in tissue distribution is assumed to be the most prominent determinant for this pharmacokinetic behavior.

8.3.1 OLIGONUCLEOTIDE ABSORPTION

PS ODNs are hydrophilic, have a relatively large molecular weight, and have multiple charges at physiological pH. These factors together with a limited stability in the gastrointestinal tract due to nuclease digestion result in a generally very low oral bioavailability, ranging from 1 to 3%. Ongoing studies, however, indicate that oral bioavailability can be increased by the appropriate release of drug and permeability enhancing excipients.⁶³ PS ODNs have also been successfully administered via subcutaneous, intradermal and pulmonary application routes.⁶²

8.3.2 OLIGONUCLEOTIDE DISTRIBUTION

After intravenous administration PS ODNs follow generally two-compartment characteristics and are rapidly cleared from plasma, predominantly via distribution processes with a half-life of 0.5 to 1.5 hr depending on the dose.⁶⁴ The terminal elimination in plasma seems to parallel that in tissues, but has due to the usually relatively low plasma concentrations (< 1 ng/mL) only rarely been assessed.⁶⁵ Since the distribution phase comprises generally more



FIGURE 8.4 Sequence–independent pharmacokinetics of phosphorothioate oligonucleotides. Average plasma concentration–time profiles of intact oligonucleotides with different sequence (ISIS2503, ISIS5132, ISIS3521, ISIS2302) during and after a 2hr IV infusion of 1 mg/kg in monkeys (n = 3-5). The profiles are nearly superimposable despite sequence differences of the investigated oligonucleotides. (From Geary, R. S., Yu, R. Z., and Levin, A. A., *Curr. Opin. Investig. Drugs.*, 2001; **2:** 563, with permission.)

than 95% of the plasma AUC, it can be concluded that plasma clearance (1-3 mL/min/kg) is primarily a function of distribution to tissue rather than metabolism.⁶⁵ This observation supports the notion that the plasma pharmacokinetics of various ONs are generally independent of their sequence and chemistry. The ICAM-1 inhibitor alicaforsen for example has a distribution half-life of 1.0 to 1.2 hr in humans.^{66,67}

Plasma pharmacokinetics of PS ODNs are nonlinear, with a more than proportional increase in plasma AUC with dose and an increase in distribution half-life at higher doses. The HIV-inhibitor trecovirsen, for example, exhibits a disproportional increase in systemic exposure after escalating doses.⁶⁸ The extent and rate of metabolism of PS ODNs is unaltered by increased dose or repeated dosing.⁶⁹ Distribution to organs, however, seems to saturate as dose increases. Thus, the nonlinearity seems to be the consequence of saturation of distribution rather than elimination processes.⁶¹

After intravenous administration, PS ODNs are detected in nearly all tissues and organs except for the brain and testes, suggesting significant transport barriers in these tissues. The extent of tissue uptake is dependent on the dose amount as well as dose rate. Major accumulation of PON occurs in liver and kidneys, and to a lesser extent in spleen, bone marrow, and lymph nodes, which seems to be independent of PON sequence. Chemical modification of the phosphorothioate backbone structure, however, may alter protein binding and organ distribution. The mechanisms for uptake into target cells has not been fully elucidated yet, but these processes are energy-, temperature-, and time-dependent, and include most likely pinocytosis and podocytosis.⁷⁰

PS ODNs are highly bound to plasma proteins, with more than 96% binding at clinically relevant concentrations, for example for ISIS 2503.⁶⁹ This high binding was present in humans as well as mice, rats, and monkeys, with little change within the range of 1 to 68 μ g/mL. Major binding proteins are α_2 -macroglobulin and albumin. The high plasma protein binding is a major determinant in the pharmacokinetics of PS ODNs. High plasma protein binding, for example, protects PS ODNs from renal filtration, so that urinary excretion of intact compound is only a minor elimination pathway.⁷¹ Plasma protein binding was saturable at very high doses in rodents with concentrations many fold higher than those used clinically, resulting in an increase in the unbound fraction and thus renal excretion of the ODNs.⁶²

8.3.3 OLIGONUCLEOTIDE ELIMINATION

While PS ODNs are relatively rapidly removed from blood, predominantly by distribution and uptake into tissues, their residence time in tissues of all examined species was found to be relatively long.⁶² PS ODNs are cleared from tissues by nuclease-mediated metabolism, with half-lives that vary between 20 and 120 hr dependent on the organ or tissue. Successive removal of bases from the 3' end is the major metabolic pathway in plasma, while both 3' and 5' exonuclease excision may occur in tissues. Exonuclease metabolism in plasma and tissues is rapid, with 30 to 40% of PON having at least one nucleotide removed after 5 min in plasma. Endonuclease-mediated degradation of PON's is generally not observed.⁷⁰

Whole-organ pharmacokinetics after 24 hr is thought to present intracellular exposure, as only very little PON remained bound to extracellular components by 24 hr after injection. Thus, whole-organ clearance is assumed to parallel cellular clearance.

Tissue accumulation of PS ODNs appears to be predictable, and steadystate levels are consistent with first-order pharmacokinetics achieved after approximately five tissue elimination half-lives.⁶²

Urinary excretion is a major route of excretion for PS ODNs, regardless of sequence or chemical structure, with the majority being shorter length metabolites rather than unchanged parent drug.⁶⁴ Urinary excretion was found to be nonlinear, with a greater fraction excreted at higher doses. Potential mechanisms include saturation of plasma protein binding as well as tubular reuptake mechanisms. Only a minor fraction of the dose is excreted into feces although enterohepatic recirculation has been suggested.⁶¹

8.3.4 SECOND GENERATION PHOSPHOROTHIOATE OLIGONUCLEOTIDES

Second generation PS ODNs have chemically been further modified for an improved stability towards nuclease activity. ISIS 104838 is a second

generation ODN aimed at the inhibition of the expression of tumor necrosis factor- α (TNF- α). ISIS104838 contains five 2'-O-(2-methoxyethyl) modified (2'-MOE) nucleosides at the 3'- and 5'-terminus, respectively. The pharmacokinetic pattern for this second generation PS ODNs is similar to first generation PS ODNs, except for a less pronounced nonlinearity in systemic exposure and a substantially prolonged terminal half-life of 27 ± 3.8 days, most likely due to the complete blockade of exonuclease digestion by the MOE modification.⁷²

8.4 PHARMACOKINETICS OF PLASMID DNA

In comparison to proteins and oligonucleotides, much less is known about the pharmacokinetics of recombinant plasmid DNA used like a "drug" in the novel treatment approach of gene therapy. Physicochemical characteristics, especially a strong negative charge and a high molecular weight, are major determinants for the in vivo disposition of plasmid DNA (pDNA) and its complexes.⁷³ After intravenous administration in rats, pDNA was detected in all major organs including lungs, liver, kidney, and spleen. Although pDNA was also detected in low concentrations in the brain,⁷⁴ the authors argued that this low level brain exposure was most likely an artifact from residual blood in the brain tissue, given that pDNA is unlikely to cross the blood-brain barrier. pDNA was observed to be rapidly eliminated from the plasma after intravenous administration in mice. Major elimination processes include rapid degradation by nucleases as well as extensive uptake into the liver. The hepatic uptake clearance actually approaches liver plasma flow, its maximal possible value. pDNA is preferentially taken up by the liver via receptor-mediated processes, preferentially by nonparenchymal cells such as Kupffer and endothelial cells.75-77

A recent study investigated the disposition kinetics of three functional forms of pDNA in rats, supercoiled, linear, and open circular pDNA. Supercoiled pDNA rapidly disappeared from plasma with a half-life of 0.15 min. Approximately 60% of supercoiled pDNA is degraded to open circular pDNA, which is subsequently nearly completely converted to linear pDNA. Conversion of open circular to linear pDNA followed Michaelis–Menten kinetics, while linear pDNA was removed with a half-life of 2.1 min. The authors suggested that the slower elimination of open circular and linear pDNA compared to supercoiled pDNA may be related to a stronger interaction of open circular and linear DNA with plasma macromolecules that might offer some protection from plasma nuclease degradation.⁷⁴

8.5 CONCLUDING REMARKS

In analogy to conventional small molecule drugs, protein and nucleotide-based therapeutics are characterized by well-defined pharmacokinetic properties that form the basis for the design of therapeutic dosing regimens as well as drug delivery strategies. Potential caveats and pitfalls, however, may arise from their similarity to endogenous and/or dietary molecules with which they share common disposition pathways. Additional resources may be necessary during the drug development process to overcome some of the obstacles arising from this similarity between therapeutic agents and body and/or food components. Advantages, however may be that therapeutic agents within one class, e.g., phosphorothioate oligonucleotides, have comparable pharmacokinetic properties that are well-predictable for newly designed members in this class. In addition, allometric scaling of pharmacokinetic parameters for protein and oligonucleotide drugs obtained in different animal species has frequently proven to be much more precise and reliable than for traditional, small molecule drugs, probably because of the fact that the handling of proteins and oligonucleotides is well preserved between different mammalian species.

The widespread application of pharmacokinetic concepts in drug development has repeatedly been promoted by industry, academia, and regulatory authorities.^{4,79} It is believed that the application of PK/PD-based concepts in all preclinical and clinical drug development phases may substantially contribute to a more scientifically-driven, evidence-based development process. Thus, in-depth knowledge of a compound's pharmacokinetic characteristics will also for protein- and nucleotide-based therapeutics continue to form a cornerstone in a rapid, cost-efficient, and successful drug development program.

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9 *In vivo* Fate of Polymeric Gene Carriers

Dagmar Fischer

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9.1 INTRODUCTION

Human genome project has substantially increased our knowledge in the various aspects of gene therapy during the last decade and opened the door for

discovery of new nucleic acid-based therapeutics. However, the therapeutic efficiency of DNA- or RNA-based drugs is mainly hampered by their short biological half-lives. A challenge for pharmaceutical scientists, therefore, is to develop site-specific controlled delivery systems, which can protect nucleic acids from rapid degradation under physiological conditions and simultaneously protect the host from their undesired side-effects. An ideal vector system should (i) be stable in the biological fluids, especially during the circulation; (ii) resistant to degradation by nucleases; (iii) capable of efficient and selective recognition by the target cells and tissues; (iv) able to penetrate the target cell membranes and gain access to the intracellular target structure, (v) efficient endosomal release and nuclear translocation; (vi) nontoxic and nonimmunogenic; and (vii) easy to produce on a large scale.^{1–3}

Compared to viral vectors and cationic liposomes, polymers have many advantages as carrier molecules for DNA and RNA delivery. They can be custom-designed for specific therapeutic needs by tailoring their molecular weights, hydrophobic/hydrophilic balance, solubility and stability of the complexes as well as binding to specific cells or tissues. Polymers are relatively safe compared to viral vectors and lipids, and their synthesis can be scaled-up for the industrial production rather easy and inexpensive.^{4,5}

Although various polymeric gene delivery systems have been investigated extensively in vitro, little attention is given to their *in vivo* use. Many polymeric gene delivery systems were effective *in vitro* but did not work *in vivo*.² Since most polymeric gene carriers are intended for *in vivo* applications, thorough understanding of their *in vivo* behavior and major obstacles to their effectiveness *in vivo* is essential. The most frequently used cationic polymers for *in vivo* gene delivery are polyethylenimine (PEI), poly-L-lysine (PLL), dendrimers and chitosans which will be discussed in this chapter.

Most published reports have focused on the quantification of gene expression as the last step of the transfection process using reported genes, such as luciferase and green fluorescent protein (GFP). However, these investigators determined gene expression mainly in the target organs or cells.⁶ However, there are some reports on the biodistribution of radiolabeled^{7,8} or fluorescent labeled⁹ DNA. Since most nucleic acids are fairly unstable in the bloodstream, biodistribution studies using these labeled nucleic acids are carried out only for a short period. Southern blots¹⁰ and polymerase chain reaction⁶ are often used to determine both the quantity as well as stability of DNA in different organs. However, there are only a few reports on the *in vivo* fate of polymeric gene carriers, although information on their organ accumulation, excretion and half-life is important especially with regard to their repeated, long-term applications.

The fate of a drug after *in vivo* administration is determined by a combination of its biodistribution, metabolism, and elimination. After administration by an extravascular route additionally its absorption has to be taken into consideration. Although drug delivery systems influence the *in vivo* fate of a drug mainly on the level of distribution within the organs, all other

steps can also be affected. The results of *in vivo* behavior depends on the physicochemical properties of both the polymeric carrier and the drug.¹¹

9.2 **BIODISTRIBUTION OF NUCLEIC ACIDS**

Biodistribution of plasmid DNA, oligonucleotides (ODNs) and ribozymes is usually determined after radiolabeling these nucleic acids with ³²P, ³⁵S, ³H or ¹²⁵I (Table 9.1).^{7,8,12} Following systemic administration, the radioactivity derived from plasmid DNA and ODNs is rapidly eliminated from the circulation, with the plasma half-lives of less than 5 min for both phosphodiester ODNs and plasmid DNA.^{6,12–15} Ribozymes are less stable than plasmid DNA with the half-life of less than 6s in human serum.¹⁶ Several mechanisms were identified which contribute to the rapid disappearance of DNA and RNA from the circulation including rapid degradation by enzymes, opsonization, and phagocytosis, or filtering by capillary beds. However, the fast clearance from the circulation may be rather due to metabolic degradation than due to the extensive uptake by the cells and tissues.^{6,15} DNA is known to get degraded by endo- and exonucleases within 5-10 min.¹⁶⁻¹⁸ For ODNs degradation was mainly accomplished by 3'-exonu--exonucleases, but also destabilization from the 5'-end was reported.¹⁸ In the case of ribozymes, the 2'-hydroxyl groups of pyrimidines were detected as the primary site of ribonuclease action.¹⁹

Following systemic administration, free plasmid DNA was mainly accumulated in the liver and excreted by the hepatic pathway. Plasmids were taken up by liver nonparenchymal cells such as endothelial and Kupffer cells via a specific scavenger receptor-like mechanism.¹⁷ The scavenger receptors are reported to recognize a wide range of polyanions since

TABLE 9.1In vivo distribution of gene drugs after intravenous application [according toRefs 6 and 12–19]

	Plasmids	Oligodeoxynucleotides (DNA, Phosphodiester)	Ribozymes
Pharmacokinetics	Two-compartmental Bi-exponential	Two-compartmental Bi-exponential	n.d.
Plasma half life time	< 5 min	< 5 min	< 6 sec
Accumulation	Liver (nonparenchymal cells)	Liver (parenchymal and non-parenchymal cells), kidneys	Liver, kidneys
Degradation	Endo-, exonucleases	Mainly 3'-exonucleases, endonucleases	Ribonucleases at 2'-hydroxyl groups of pyrimidines
Major elimination pathways	Liver	Kidneys, liver	Kidneys, liver

pre-administration of polyanions was found to reduce the liver uptake of naked DNA. In contrast to plasmids, for ODNs a nonspecific liver uptake in both parenchymal and nonparenchymal cells could be observed.¹⁷ While plasmids do not undergo glomerular filtration, for oligonucleotides both hepatic and renal pathways were identified as the main routes of excretion. Fifteen minutes post-administration, almost 15–20% of the injected ODNs accumulated in the liver and kidney, whereas all other tissues had less than 1% each. Miyao et al.²⁰ and Sawai et al.²¹ described that single-stranded ODNs undergo glomerular filtration, but they are also subject to tubular reabsorption via a scavenger receptor-like mechanism and to uptake from the capillary side.

Although nucleic acids have many potential therapeutic applications, they have limited clinical use because of their low stability, fast metabolism, and excretion under physiological conditions. Although both DNA and RNA have been chemically modified to improve their *in vivo* stability,^{15,22} there are still un-solved problems related to their low cell uptake and nonspecific transport and toxicity to nontarget cells. To avoid their recognition by scavenger receptors after systemic administration, the anionic charges of these nucleic acids should be masked.

9.3 SYSTEMIC ADMINISTRATION OF POLYMER/DNA COMPLEXES

Polyplexes can be delivered to disseminated and widespread disease targets or metastatic nodules via systemic administration. However, systemic gene delivery faces many obstacles, such as anatomical size constraints, interactions with biological fluids and extracellular matrices as well as binding of polmeric carriers to various nontarget cell types. The carrier system has to ensure (i) the drug accumulation at the intended sites, (ii) rerouting drugs from sites of toxicity, (iii) and increasing the plasma half-life of labile or rapidly eliminated drugs. The biodistribution and elimination patterns of injected drug delivery systems are dictated mainly by their overall physicochemical properties. Size and stability of the complexes, hydrophobicity of the particle surface as well as density and type of the surface charge were found to be the key parameters for their plasma half-lives, extravasation through endothelial gaps as well as adsorption of opsonizing proteins onto the surface of carrier systems and subsequent uptake by the reticuloendothelial system (Figure 9.1).

9.3.1 CIRCULATION IN THE BLOODSTREAM

Following systemic administration, ideal polyplexes should (i) remain stable without dissociation to protect nucleic acids from degradation, (ii) be soluble without aggregation, and (iii) not interact nonspecifically with cellular blood components, vessel endothelia, and plasma proteins. The most important properties, which contribute to these aspects are particle size of the



FIGURE 9.1 In vivo fate of polymer/DNA and RNA polyplexes after systemic administration.

polyplexes, molecular weight as well as surface charge of the polymer, and the charge ratio of the polyplexes.

After intravenous injection particles greater than 5 to 7 μ m in diameter are often accumulated in the lung capillaries, while particles with a diameter of less than 5 μ m are generally cleared from the circulation by the cells of the reticuloendothelial system. Particles in excess of 7 μ m are larger than the blood capillary diameter (about 6 μ m) and will be mechanically filtered.²³ The disposition of particles in the pulmonary capillary beds can result in occlusion of capillaries and may be one of the leading pathogenic mechanisms for lung embolism. The lung capillary bed is the first to be reached after intravenous application. Accordingly, larger DNA complexes showed higher lethality (about 50%) than smaller ones (15%).²⁴ Although nonviral vector systems are usually characterized by sizes in the range of 50 to 200 nm, the aspect of size is important with regard to the tendency of cationic carriers to form aggregates.

The major problem associated with gene delivery systems are their numerous nonspecific interactions during circulation due to their positively charged surface. After tail vein injection, high concentrations of polyplexes were found to adhere at the injection site, mainly due to interactions with the negatively charged vessel surfaces. This effect could not be observed after injection of polyplexes into larger vessels such as jugular vein.²⁵ Electrostatic interactions of polycations such as polyethylenimine (PEI), poly(L-lysine)

(PLL), and dendrimers with the negatively charged surfaces of blood cells especially erythrocytes were found to result in the formation of large aggregates. The tendency to form erythrocyte aggregates and subsequent systemic toxicity increased with increase in the molecular weights of polycations as well as net cationic charges of the complexes. PEI of 22 kDa showed less aggregating effects than 800 kDa PEI and was better tolerated *in vivo*. Complexes with a net positive surface charge were characterized by higher aggregation, acute toxicity, and lethality than the neutral one ^{10,26,27}

Interactions with plasma proteins were also reported to influence the circulation time and cellular uptake of polyplexes. After intravenous injection of positively charged transferring–PEI/DNA complexes, there is often acute lung embolism and in up to 50% lethality of treated mice.²⁴ Albumin, the most important serum protein, appeared to be the major protein bound to polycation/plasmid complexes, presumably through hydrophobic interactions rather than simply electrostatic binding. Although albumin was not able to dissociate polycation/DNA complexes and release plasmid DNA, it induced the formation of a ternary system with a reversed surface charge and the formation of aggregates which were captured by scavenger receptors of the liver or accumulated in the lung capillary beds.^{7,28–30}

Beside aggregation, interaction of polyplexes with plasma proteins can also induce decomposition of the delivery systems, release and subsequent degradation of DNA and RNA. Compared to free DNA which was substantially degraded in mouse, rat, and human blood by the activity of nucleases, there is significantly less degradation of DNA due to little dissociation of plasmid from the complexes in the bloodstream.^{6,7,15,18} The stability of the complexes in physiological fluids was dependent on the physicochemical characteristics of the polycation used for complex formation with the DNA as well as the net charge of the complexes. Complexes of plasmids with low molecular weight polylysine displayed increased aggregation in salt solutions as well as lower relative solubility within the blood compared to the complexes based on polylysine of 10-fold higher molecular weight.¹⁵ Weaker DNA binding of the low molecular polycations and subsequent formation of less compact structures were held responsible for these effects. The ability to resist the influence of salts and to protect DNA from enzymatic degradation was directly correlated with the strength of DNA binding and increased with higher polymer to DNA ratios of the complexes.³¹

Blood and plasma concentration/time-curves of plasmids or ODNs complexed with different polycations followed a two-compartmental model with a rapid decrease in a steep α distribution phase, followed by a slower β elimination phase for both the polymer and DNA.^{6,7,15,25} In case of free DNA, there was rapid clearance of the DNA from the bloodstream because of their fast enzymatic degradation,⁶ but for polymer-complexed DNA there was an extensive tissue entrapment.^{7,25}

When the pharmacokinetic profile of naked DNA was compared to complexed DNA, the polycations decreased the clearance of the DNA from the circulation (Figure 9.2). Subsequently, complexes were characterized by a


FIGURE 9.2 Plasma/concentration time course of ODN/25 kDa PEI complexes ($^{125}I =$ squares, $^{32}P =$ triangles) at N/P 6 (1 µg PEI/mouse) up to 2 h after intravenous injection. Data are means \pm SE of four mice and expressed as percent of injected dose per ml. The plasma concentration/time profile of free ODN was inserted for comparison. 103

higher mean residence time in the blood compared to DNA as indicated by an increase in AUC.^{12,15} The effects of the polycations on pharmacokinetics were more pronounced in the β phase than in the α phase. The distribution half lives of free and polymer-complexed plasmids were in a comparable range, e.g., with 0.7 min and 0.9 min, respectively, for PEI complexes. In contrast, the elimination half life of plasmids and ODNs complexed with PEI or polylysine were several fold higher compared to free DNA.⁶ Similar pharmacokinetic profiles were observed for different types of polymers such as PEI and poly (L-lysine) as well as for different types of DNA and RNA.

Double labeling experiments of 25 kDa PEI and ODNs revealed that the blood concentration levels of both components were similar within the first few minutes. However, differences between ODN and polycation could be observed after time points >15 min suggesting that desintegration began within this time range after IV injection. Furthermore, a comparison between the AUCs of the polycation in blood and plasma revealed that about 50% of 25 kDa PEI appeared to be bound by blood cellular elements.

9.3.2 EXTRAVASATION

One of the obstacles which has to be overcome after systemic administration is the extravasation of polyplexes beyond the endothelial barrier lining the blood vessels. Three main types of endothelia are distinguished due to the fine structure and continuity of their endothelium and the basal lamina: continuous, fenestrated, and discontinuous.³² Extravasation of both naked and complexed DNA via continuous (complete endothelia and basal lamina) or fenestrated (endothelia with fenestrae closed by diaphragms) capillaries was found to be very low due to the tightness of the endothelial barriers and the huge size and high molecular weight of plasmid DNA and its complexes. Only organs and tissues with the discontinuous type of capillary walls such as liver, spleen, and bone marrow allowed the penetration of small carrier systems. These capillaries are characterized by a discontinuous or absent basement membrane, and endothelial cells perforated by fenestrations which can be passed by molecules, nanoparticles and interpolyelectrolyte complexes in the range from 100 to $1000 \text{ nm.}^{23,33}$

In pathophysiological states, such as tumors and infected or inflammed sites defective endothelia with large meshes exists and make the movement of macromolecules and particles to the interstitial fluids possible.^{34,35} Consequently, the permeability of the endothelial barrier and the size, flexibility and deformability of the polymeric carriers are important parameters for extravasation of particles. The fenestrated glomerular membranes additionally restrict the movement beyond the endothelial barrier by charge, since positively charged macromolecules showed higher glomerular permeation than anionic macromolecules of similar size.^{2,33}

9.3.3 **Opsonization and Clearance by RES**

The major obstacle in the systemic use of polymeric carriers is the so called "RES clearance." This means that after intravenous injection particulate systems will be recognized as foreign by the body and rapidly cleared from the bloodstream by phagocytic macrophages of the reticuloendothelial system (RES) of the liver (Kupffer cells) and spleen. This is a very efficient and fast process since already after 5 min about 60 to 90% of particulate carriers were found to be removed from the circulation leading to very shortplasma half-lives.^{2,32,36} The recognition of particles by RES is mediated by interactions of blood components with the artificial surfaces of the carriers (opsonization) leading to activation of the complement system.^{7,37} Activation can take place via "classical" pathways based on antigen-antibody reactions or "alternative" pathways. The alternative pathways which are manly triggered by adsorption of C3b complement protein onto the surface of the polymeric carriers were found to be an important way in the processing of particles.²³ An enhanced RES-uptake was also observed after interaction of intravenously injected polymeric particles with other blood components, such as serum albumin, fibronectin, fibrinogen, and immunoglobulins.24,30

The particle size and surface charge are the two most important physicochemical properties of polymeric carriers, which determine the degree of their uptake by the RES.^{6,15,38} The gaps in the sinusoidal capillaries of the liver are about 100 to 200 nm in diameter and therefore, complexes must not be greater than 200 nm to reach the hepatocytes. However, most of the particles were found in Kupffer cells of the liver. The uptake of particles by the liver and spleen due to opsonization and phagocytic activity was found to increase with higher particle sizes. Larger particles were cleared faster than smaller particles.

The surface charge is known to affect the clearance of particles from the blood.^{23,24,37} Negatively charged particles are known to be cleared from the bloodstream relatively slower than positively charged systems, while neutral particles exhibited an increased circulation half-life. Noncharged particles showed *in vivo* low binding of complement factors and consequently, low uptake by the RES. Highly positively or negatively charged particles had a high tendency to accumulate in the liver. Cationic systems accumulated in the liver due to their large surface areas permitting adhesion of the cationic particles with the negatively charged cell surfaces. By comparison, negatively charged particles are taken up by Kupffer cells by scavenger receptor-mediated endocytosis.⁷

Additionally, the degree of RES-uptake can be correlated with the ability of the carriers to activate the complement cascade. A strong correlation between the density of accessible positively charged surfaces and the extent of complement activation was reported. Large polycations such as 25 kDa PEI or high generation dendrimers showed a high potential in activating the complement system, while small polyamines such as oligo(L-lysine) exhibited this effect to a lesser extent.³⁹ After complex formation with DNA, neutral complexes exhibited only low levels of complement activation. In contrast, high charged complexes were subjected to adsorption of opsonins such as IgG which contributed to a rapid clearance.⁷ The RES clearance after systemic administration is the major obstacle against targeting to specific cells or tissues other than the liver and spleen or formulation of the long circulating systems.

9.3.4 ORGAN DISTRIBUTION

Complex formation and encapsulation of DNA and RNA with polymers induced a significantly higher and prolonged organ retention compared to naked DNA, depending on the structure of the polymers and the physicochemical characteristics of the complexes.^{6,7,12,15} This was attributed to enhanced protection and stability of complexed DNA from nucleases and other degradative elements in the blood and tissues. Biodistribution of unmodified gene carriers of different cationic polymers, such as poly(Llysine), PEI or dendrimers was quite similar after intravenous administration. The characteristics of these polycations were found to be an important parameter for the organ distribution of DNA and RNA. The type of gene drug did not significantly influence the organ distribution profiles.

We observed in double-labeling experiments an equal distribution of ³²P-labeled ODN and ¹²⁵I-labeled polycation after 15 min in all organs which can be interpreted as evidence for organ uptake of intact complexes, indicating partial stability during the distribution phase (Figure 9.3.A). At 30 min after systemic administration of PEI/plasmid complexes, intact and also partially degraded DNA could be detected as analyzed by Southern blotting.¹⁰



FIGURE 9.3 Comparison between organ distribution of free ODN and polyplexes formed by ODN and 25 kDa PEI at a dose of 1 µg polyethylenimine and N/P-ratio 6 at 15 min (A) and 12 h (B) after injection. The distribution profile of the ³²P-signals was compared to that of the ¹²⁵I-signals. Values are given as the mean \pm SE of four animals as percent of injected dose per organ.¹⁰³

At 15 min post injection, the amount of radioactivity acculated in different orgarns were comparable for DNA and polycation (Figure 9.3B). However, there were significant differences at 2 and 12 h post injection. Biodistribution of the polymer signal remained constant over time, whereas there was decrease in the radioactive signals of ODNs over time, presumably due to ODNs release from the complexes followed by their degradation by nucleases and elimination by the liver and kidney.

In the initial phase of distribution, the delivery of the complexes from circulation to organs is a roughly unidirectional process.^{17,25} In many reports already the highest accumulation of DNA and polymer at 15 to 30 min after systemic administration was found in the liver.^{6,7,12,15} In contrast to free plasmids and ODN which were also distributed in the liver, the levels of hepatic accumulation of the DNA were several times higher after complexation, e.g., seven times for plasmid/25 kDa PEI complexes.⁶ Since the fenestrations in the endothelia of liver and spleen are believed to be smaller than the size of the injected complexes, opsonization and rapid clearance by phagocytic

macrophages were held to be responsible for the accumulation. Histological investigations confirmed the uptake of complexes in Kupffer cells while no uptake could be observed in hepatocytes.¹⁵ Comparison between organ distribution of plasmid DNA and gene expression revealed significant differences.¹⁰ Although intact plasmid was mainly deposited in the liver, the transfection efficiency of polymer/plasmid complexes was comparably low. Several authors reported that a high plasmid accumulation in liver did not result in high gene expression due to the role of liver in removal of foreign particles from circulation mainly by Kupffer cells and therefore, rapid degradation of DNA.^{10,12,39}

Beside the liver and spleen, the lung was identified as one of the main organs of gene expression after systemic injection of polymer-based complexes, possibly accomplished by aggregate formation of the complexes with blood components and subsequent trapping of the aggregates in the lung capillary bed.²⁹ The highest differences in disposition between free DNA and polymer-complexed DNA were observed for the lungs. PEI-complexed plasmids showed more than two orders of magnitude higher AUC values in the lung than naked DNA.⁶ With higher doses of DNA a substantial increase in the lung accumulation could be detected. Gene expression in the lung after systemic application was found to be rather similar to organ distribution.¹⁰ In comparison with all other ogans, the highest levels of gene expression were found in the lung which was not only attributed to a high disposition but also to a more efficient expression of genes. The reason for the higher ability of the lung tissue to translate plasmids to proteins can only be speculated. Although the lung capillary bed is characterized by a tight endothelium, a rapid crossing by complexes was detected which was attributed to specialized transporters for polyamines, the more fragile vasculature in the alveoli allowing passage of small particles as well as specialized forms of transcytosis.40-42

Compared to the results of systemic application of free DNA, the amount of polymer-complexed gene drugs measured in kidneys was comparatively low suggesting that DNA within complexes was protected from degradation and subsequent renal excretion. Other tissues such as heart, cortex, and fatty tissue did not exhibit significant levels of DNA or the polymer.^{6,10,12,25}

9.4 LOCAL APPLICATION OF POLYPLEXES

Arterial vessels,^{43,44} muscle,⁴⁵ lung,^{46–49} tumor,^{27,48,50–52} brain,^{53–59} kidney,⁶⁰ heart,⁶¹ and skin^{62,63} are used for local aplications of polymer/DNA and RNA complexes. The target sites are easily accessible and several barriers to systemic application can be avoided. Either by direct injection or via the catheters, high concentrations of the complexes can be delivered to the target sites. The following sections describe the most frequently used local applications of polycation/gene drug complexes.

9.4.1 INTRATRACHEAL AND AEROSOL ADMINISTRATION

For the therapy of genetic and acquired pulmonary diseases, the lung is an ideal candidate for gene delivery due to its large surface area, thin air–blood barrier, and relatively low enzyme content.⁶⁴ Lung tissue can also be accessed by local intratracheal and aerosol administration depending on the cell type being targeted and the function of the transgene product.⁶⁵ However, the innate immunity of the lung surfaces, including an adhesive mucus layer in the upper respiratory tract, surfactant proteins that function specifically in host defense, and alveolar macrophages in the deep lung, provides a barrier to gene delivery. Furthermore, the epithelial barrier hinders the uptake or passage of gene carriers by intercellular tight junctions, actin-deposition, and structure of the glycocalyx on the surface as well as the rate of endocytosis.⁶⁶

At 24 h post-intratracheal instillation of PEI/plasmid complexes, reporter gene expression was about 12-fold higher than in rats treated with free DNA.⁶⁷ However, instillation induced unequal and heterogeneous gene expression due to insufficient spreading of polyplex solutions.^{68,69} Compared to the intra-tracheal instillation, aerosol delivery of polyplexes into the lungs is not only more convenient but also more efficient technique. In contrast, aerosolization leads to more uniform distribution of gene expression.^{67,70,71} Aerosol delivery of p53 plasmid/PEI-complexes to mice resulted in significant reductions in the numbers and size of lung tumors and prolonged the survival time of animals.⁷²

The type of polycations, applied dose, particle size, and surface morphology and charge of polyplexes have significant influence on biodistribution and transfection efficiency. PEI/plasmid complexes strongly interacted with bronchoalveolar lavage fluids and were inhibited by alpha(1)glycoprotein and mucin in a dose-dependent manner, most likely due to changes in surface charge. In contrast, fractured dendimer/plasmid complexes showed little influence by bronchoalveolar lavage fluids.⁷³ Nevertheless, PEI was found to be superior to the dendrimers in transfecting cells.^{67,73} PEI-based complexes revealed a more rapid onset of gene expression than chitosans and many other cationic polymers, possible due to its protonsponge effect leading to a faster endosomal release of the complexes.⁷⁴ When gene expression in the lung after nebulization of PEI/plasmid complexes was optimized with regard to dose and composition of the complexes, the highest expression could be observed 24 h post administration. Almost 40-50% of the peak levels were still observed one week after nebulization.⁷⁵ Particles larger than 5 µm were not phagocytosed by alveolar macrophages, and complexes smaller than 260 nm were not detected by macrophages. Particles with hydrophobic or charged surface chemistry were more likely to be phagocytosed than those with neutral or hydrophilic properties. Clearance of particles by macrophages in the lungs could be avoided by coating with hydrophilic carriers or surfactant components such as dipalmitoylphosphatidylcholin.76,77

9.4.2 DIRECT APPLICATION TO CENTRAL NERVOUS SYSTEM (CNS) AND BRAIN

Movement of polymeric particles from circulation into the brain tissue is highly resticted by particular morphologic and enzymatic features of the brain capillaries. Brain endothelial cells lack fenestrations, have few pinocytotic vesicles and form very tight junctions which are responsible for the formation of a blood–brain barrier effectively restricting the movement of most molecules from blood to brain. Additionally, brain capillaries are completely surrounded by astrocytes, which contribute to the barrier properties.^{78,79}

To overcome the limited access of gene drugs to the brain, until now only the strategy of receptor-mediated endocytosis after systemic administration to cross the brain endothelium was sucessful. Gene delivery systems linked to monoclonal antibodies against the human insulin receptor or the rat transferrin receptor (OX26), located on the surface of brain endothelial cells, underwent transcytosis across the blood-brain barrier and were able to reach the brain side.^{80,81} Most of the protocols focused on local applications of plasmids and ODNs by intraventricular, intrathecal, intracarotid, or localized regio-specific injections.^{53–55,59} Significant levels of gene expression and sometimes long lasting effects over 4 to 6 days could be observed in all experiments. A single injection of PEI/plasmid complexes into the lumbar subarachnoid space induced 40-fold higher expression in the spinal cord than naked DNA.⁵⁹ Transgene expression persisted for about 5 days. Intraventricular injection of 22 kDa PEI/plasmid complexes showed a dose-dependent increase in the number of transfected cells and a wide spread pattern of expression along the ventricle which maintained over 6 days.^{54,57}

Transfection efficiency of polymeric gene delivery systems is dependent on the physicochemical properties of the polycations used for complex formation with plasmid and the charge ratio of polymer/plasmid complexes.⁵⁸ PEIs of 22–25 kDa worked best in the central nervous system.⁵³ The optimal ratio of polymer to DNA and consequently, the net charge of the complexes, can vary according to species and brain region targeted. For PEI-based complexes in mouse brains a polymer to DNA charge ratio of 6 forming slightly positive complexes provided the best transfection results whereas in adult rats substantia nigra, complexes with charge ratio of 3 were found to be optimal.⁸²

9.4.3 INTRATUMORAL INJECTION

For tumor therapy, different applications such as injections in the tumor supplying blood vessels, intratumoral administration as well as intraperitoneal injections of gene delivery systems have been reported. The most effective route to reach neoplastic cells is the administration of polyplexes into the artery feeding the tumor, since it provides the drugs access to the rapidly growing tumor areas.⁸³ Tumors blood vessels are characterized by higher

permeability than healthy tissue and facilitates the extravasation of drug carrier systems.^{34,35} However, tumor vasculature is often highly un-organized with tortuosity, disarray of vessels, and occlusion of vessel lumens which hinders homogeneous drug disposition throughout the tumor. Moreover, a high degree of collagen-rich matrix in solid tumors, a higher pressure in the tumor than in the circulation, and necrosis in certain areas make a homogeneous distribution of carriers more difficult.⁸³ Conclusively, the particle size of the complexes is a key parameter for their passage through the tumor endothelial gaps which are in the range of several hundred nanometers. The degree of carrier uptake and distribution in the tumor can be modified by their particle size and surface charge, since highly cationic particles were found to preferentially deposit in the regions of high angiogenic proliferation.⁸⁴

For intratumoral application, particle size, charge and concentration of polyplexes as well as the speed of injection are important for diffusion of the complexes in the tumor and subsequent gene expression.⁵² Gene transfer of transferrin-PEI/plasmid complexes in subcutaneously growing tumors was 10 to 100-fold more efficient than the treatment with naked DNA.²⁶ Treatment of hepatomas in mice with PEI/suicide gene complexes significantly suppressed the tumor growth and prolonged the survival times of the animals.⁸⁵ However, due to the unusual anatomical features of the tumors. some limitations were observed. After intratumoral injection of galactosylated PEI/DNA complexes in head and neck carcinoma, gene expression was detected only in proliferating cells of the tumor periphery. In necrotic and keratinized areas neither gene expression nor diffusion of the carriers could be observed.⁸⁶ To achieve transfection toward the total tumor mass, poor diffusion could be overcome by infusion of complexes using a micropump. Linear PEI/DNA complexes applied with a pump demonstrated a higher and prolonged gene expression compared to complexes which were injected with a syringe.52

Intraperitoneal injection of complexes was used for the local therapy of peritoneal disseminated tumor cells occurring at advanced stages of pancreatic, gastric or ovarian cancers. A linear PEI/plasmid complex was found to be more efficient than lipidic formulations with a certain specificity of gene expression in tumor cells.⁵¹ Intraperitoneally injected low molecular weight PEI/ribozyme complexes displayed a prolonged blood circulation time as well as a higher accumulation in a subcutaneous tumor compared to naked ribozyme 60 min after administration.⁵⁰

9.5 STRATEGIES TO INCREASE EFFICIENCY OF POLYPLEXES

As discussed above, pharmacokinetics and organ distribution of particulate delivery systems are influenced by their surface characteristics. Therefore, precise control of the particle size and surface properties of polyplexes is essential for proper regulation of their interactions with proteins and cells, to allow extravasation, to avoid deposition in the lung or uptake in the organs of the RES, and to achieve a targeting to the site of interest.

9.5.1 STERIC STABILIZATION OF POLYPLEXES

To improve the physicochemical and biological stability of polyplexes and to change the *in vivo* distribution patterns, many second generation polymeric carriers have been designed by attaching hydrophilic nonionic and water soluble polymers such as polyethyleneglycol (PEG),^{12,24,25,87–89} transferrin,^{10,24} pluronic,^{88,90–92} or pHPMA.^{12,93} Highly mobile and hydrated polymer strands are believed to shield the surface charge of polyplex surface, sterically preventing nonspecific interactions with the biological environment and thus, prolonging their plasma half lives.⁹⁴

Two different approaches have been investigated for steric stabilization of polyplexes. On the one hand, a hydrophilic polymer is covalently attached to the cationic polymer after complex formation.^{24,93} Another strategy is the synthesis of block or graft copolymers of the condensing and shielding polymers prior to mixing with the gene drugs.⁵⁰ The major drawback of this approach compared to the first strategy is the possible hindrance of complex formation by the nonionic part of the copolymer. The structure of the second generation complexes consists of a hydrophobic, neutral core formed by DNA and polycation blocks, surrounded by a shell of the hydrophilic nonionic polymer. In all cases, introduction of nonionic polymers into the complexes reduced the particle size down to about 100 nm as well as the surface charges to neutrality and stabilized the carriers by steric hindrance against aggregation and nonspecific interactions.^{12,50,88,89}

9.5.1.1 PEGylation

PEGylation of polymeric gene carriers resulted in a decrease in disposition and gene expression in the lung with lower initial toxicities compared to unmodified complexes.^{12,24,88} This was most likely related to a lower tendency of the complexes to aggregate, decreased interactions with blood constituents and therefore, a lower rate of filtration by pulmonary capillaries. Furthermore, PEGylated carriers were also characterized by a slower uptake by the cells of the RES.^{12,50} A comparison between 25 kDa PEI and a PEGylated derivative grafted with fifty chains of 550 Da PEG (PEI(PEG)₅₀) demonstrated that 15 min after intravenous injection the PEGylated copolymer reached only 50% of the values of the nonmodified polycation in liver and spleen (Figure 9.4). Biodistribution of PEGylated PEI approached the distribution pattern of the parent compound after 2 h. This was correlated with prolonged circulation of PEGylated PEI in the blood with increased (+63%) area under the curve (AUC) and an elevated β phase compared to unmodified PEI. Using PEGylated poly(L-lysines) the amount of polyplexes circulating in the blood shifted from 15 to 69% compared to the nonPEGylated polymer/plasmid complexes.¹²



FIGURE 9.4 Organ distribution of PEGylated PEI/ODN complexes (N/P 6) given as mean \pm SE (n=4) of the percent of injected dose per organ 15 min after intravenous injection (1 µg PEI/mouse) with comparison of ³²P-signals.¹⁰³

These observations can be explained by the efficient steric barrier created by PEG chains, leading to reduction in opsonization and poor uptake by the RES. While nonmodified PEI/DNA complexes were found to adsorb plasma proteins to their surfaces, PEGylated complexes were not able to interact with IgM, fibronectin, fibrinogen, complement factor C3, and did not aggregate.²⁴ Additionally, PEGylated polyplexes were not found to aggregate erythrocytes. However, aggregation could be induced even with PEGylated complexes with an excess of polycation where a surplus of cationic charges and also free polymer was available.

In most reports, the differences in blood circulation times between modified and nonmodified carriers are quite short in comparison to the effects which were obtained, e.g., with PEGylated liposomes.⁹⁵ Furthermore, circulation times of PEGylated polycation/DNA complexes are sometimes contradictory. PEGvlation of some poly-L-lysine or PEI/DNA complexes did not result in extended plasma circulation.¹² For efficient stealth effects, the composition of the copolymers plays an important role and may explain the different results. A systematic investigation of a series of PEGylated PEIs revealed a dependency of their in vivo organ distribution and pharmacokinetic on the block length of PEG chains and the degree of substitution.⁵⁰ The highest stealth effect could be obtained using copolymers of PEI grafted with many short PEG blocks which obviously led to a more effective shielding than fewer and larger blocks. Furthermore, the stability of PEGylated complexes can be reduced by introduction of PEG., While the radioactivity of ³²P-DNA complexed with unmodified ¹²⁵I-25 kDa PEI were in comparable range in the different organs 15 min after injection, polyplexes based on PEGylated PEIs displayed a distinct pattern with the two tracers accumulated in the organs to varying degrees and a DNA-distribution profile comparable to that of naked DNA (Figure 9.4). This suggests that most of the ³²P-DNA and ¹²⁵I-PEGylated PEI must have been released from the complexes which was attributed to the fact that the electrostatic interactions between polymer and DNA were inhibited by the PEG-moiety, formation of less compact structures, and a less efficient protection against nucleases.⁹⁶ Similar observations were reported for poly(L-lysine) derivatives. Compared to unmodified polymers, substitution of poly(L-lysine) epsilon-amino sites with PEG resulted in faster degradation of polyplexes, and rapid release of DNA after IV. administration.³⁹ Although PEGylated polymers did not have the strongest binding of DNA, some of them achieved high levels of gene expression after systemic application due to an easier release of DNA from the complexes.

To further increase the stability of polyplexes during circulation, primary amines of the polycation were crosslinked by disulfide bonds which were extracellularly stable and were cleaved by reduction inside the cells. These polyplexes showed 10-fold increase in plasma circulation in mice following IV application.⁹⁷ An extended half-life in the bloodstream was also observed using multivalent PEG to induce crosslinking of the complex surface.¹²

9.5.1.2 Complex Stabilization with Poly(*N*-(2-hydroxypropyl)methacrylamide)

Covalent binding of hydrophilic, monovalent end-reactive poly-[*N*-(2-hydroxypropyl)methacrylamide] (pHPMA) to sterically modify the surface of polyplexes improved their resistance to protein binding and reduced the phagocytic uptake.⁹³ However, after systemic administration complexes were cleared from the bloodstream more rapidly and accumulated to a higher extent in the liver than the unmodified complexes. In a second series of experiments multivalent pHPMA was coated onto the surface of preformed PEI- and PLL-based polyplexes to accomplish in addition to steric stabilization a lateral type of stabilization by linking together surface amino groups.¹² The alpha half-life for bloodstream clearance could be extended by the multivalent pHPMA coating to more than 90 min compared to about 5 min of unmodified complexes.¹² *In vivo* circulation could be improved with increasing molecular weight and higher amounts of the multivalent pHPMA, as well as with higher administered doses.

9.5.1.3 Modification of Polyplexes with Pluronic

Pluronic is a block copolymer of ethylene oxide and propylene oxide. Pluronic was covalently bound to polycations, since it belonged to a class of polymeric surfactants, which are able to interact with biological membranes and enhance the transport of compounds into cells.⁹⁰ After systemic application, conjugates of PEI and Pluronic P123 decreased the transgene expression in the lung and simultanously increased gene expression in the liver compared to unmodified PEI.^{91,92} In contrast to the unmodified polycation, the complexes preferentially accumulated in the hepatocytes, suggesting their ability to cross the endothelial barrier and reach the parenchymal cells. The modification of tissue distribution compared to the unmodified systems or PEGylated carriers were suggested to be related to differences of the hydrophilic–lipophilic balance of the polyether chains grafted to PEI in these molecules.⁹² Thus, this system may potentially be used for gene delivery to the liver.

9.5.1.4 Transferrin Modification of Complexes

Ligands which were covalently bound to the polycation were able to act as a shielding barrier. Incorporation of transferrin into polymer/DNA complexes reduced significantly the cationic surface charge compared to nonmodified complexes with increasing degree of substitution.²⁶ The higher the ligand density, the more nonspecific interactions with erythrocytes and plasma proteins. Consequently, after *in vivo* administration the toxicity of the transferrin-shielded polyplexes was reduced.¹⁰

9.5.2 TARGETING STRATEGIES

To improve the delivery of polyplexes to a desired organ, tissue or cell type and to avoid undesired effects in nontarget organs, two different strategies, passive and active targeting, were investigated.⁹⁸ Considerations in the development of site-specific or targeted delivery systems are (i) the characteristics of the target site, (ii) the physicochemical characteristics of the drug and (iii) the carrier as well as (iv) the specificity of the targeting process.

9.5.2.1 Passive Targeting

The strategy of passive targeting uses particular anatomical and physiological properties of the target tissue such as permeability of their endothelia or pathophysiological modifications during disease, as well as the natural physicochemical properties of the carrier systems.⁹⁸ Gene delivery systems were found to accumulate passively in solid tumors which was related to the enhanced permeability and retention effect ("EPR effect").99 A higher leakiness and irregularity of tumor endothelia with gaps as large as several hundred nanometers, the lack of normal lymphatic drainage, and hypervascularization of tumor tissues allowed higher penetration of particulate drug carriers without specific targeting moieties. Comparable effects were also reported for other pathological situations such as infection and inflammation with more permeable endothelia.^{34,35} Passive targeting was possible for the liver and lung after systemic application. As discussed above, conjugation of Pluronic to cationic polyplexes allowed specific targeting of ODNs to hepatocytes of the liver.^{91,92} After tail vein injection, complexes of plasmids and linear 22 kDa PEI showed exceptionally high gene expression in the lung strongly restricted to type II pneumocytes with low levels in all other organs.¹⁰⁰

9.5.2.2 Active Targeting

The second approach to accumulate particulate carriers in tissues or cells of interest is the conjugation of specific homing ligands capable of being selectively recognized by the target cells. The complexes interact with the cognate receptors on the target cells to fascilitate receptor-mediated internalization by the cells.⁹⁸ Ligands of different groups such as antibodies, growth factors, carbohydrates, lectins, and integrins have been used for active targeting of DNA and RNA delivery systems. A number of ligands are listed in Table 9.2, which are used for receptor-mediated gene transfer. All targeted complexes showed a significant increase in gene expression compared to complexes without targeting moieties in the organ of interest. The most impressive targeting effects were reported for antibodies with gene expression levels which were usually higher than that of other targeting ligands. They were most preferred due to their high specificity of target binding. However, after repeated *in vivo* applications the possibility of immunogenicity has to be taken into consideration.

TABLE 9.2Most frequently used ligands for cell specific receptor-mediated targetingof polyplexes

Receptor	Ligand	Specificity	Carrier	References
Transferrin receptor	Transferrin	Various cell types	PLL, PEI,	10, 24, 104
Asialogylcoprotein	Galactose	Hepatocytes	PLL, histone	3, 17, 102, 105, 106, 107
	Lactose	Hepatocytes	PLL, PEI	
	Mannose	Hepatocytes, dendritic cells	PEI, PLL	
	Asialoorosomucoid	Hepatocytes	PLL	
Insulin receptor	Insulin	Brain endothelia	PLL	80, 81
Integrins	RGD peptides	Tumor endothelia	PEI	108
Folate receptor	Folate	Tumor cells	PEI, PLL	109,110
FGF receptor	FGF	Various cell types	PLL	111
EGF receptor	EGF	Various cell types	PEI	112
Antibodies				
CD3	Anti-CD3	T-lymphocytes	PEI	113
Polymeric immunoglobulin	Antipolymeric immunoglobulin	Respiratory epithelia Hepatic epithelia	PLL	114
EGF	Anti-EGF	Carcinoma	PLL	115
Thrombomodulin	Antithrombomodulin	Lung endothelium	PLL	116
PECAM	Anti-PECAM	Lung endothelium	PEI	117

FGF, fibroblast growth factor.

EGF, epidermal growth factor.

PECAM, platelet endothelial cell adhesion molecule.

The most common techniques to conjugate the ligands to the amino groups of polycations are (i) conjugation of aldehydes, such as reducing sugars or oxidized glycoproteins by reductive amination, (ii) isothiocyanate groups to form a thiourea derivative, (iii) binding of compounds with a carbodiimide-active carboxyl group through amide bond formation, and (iv) modification of amino groups with bifunctional linkers with a reactive ester such as *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and subsequent formation of disulfide bonds by reaction with thiol groups.¹⁰¹ The degree of substitution has to be taken into consideration, but was investigated only by few studies. A high substitution of polyplexes with transferrin induced a shielding effect *in vivo*.^{10,26} In contrast, a high degree of substitution may also create a new surface on which opsonization can occur.¹¹ Increasing the degree of carbohydrate substitution from 3.5 to 31% of all PEI amino groups compromised DNA condensation and complex stability and decreased the transfection efficiency.¹⁰²

9.6 CONCLUDING REMARKS

Polymeric carriers are promising for efficient and safe delivery of nucleic acids. After local and systemic administrations many obstacles have to be overcome to allow gene drug delivery systems to reach their target site. The colloidal, surface, and chemical properties of DNA and RNA complexes with polycations are highly responsible for controlling the extent and rate of delivery of gene drugs to cells. However, once arrived at the surface of the cells additional hurdles on the cellular level have to be overcome. Complexes have to enter the cells bypassing the cell membranes, escape the endosomal/lysosomal compartment with degradative enzymes, traffic through the cytoplasm, and enter the nucleus. The physicochemical characteristics of polyplexes, such as size, charge, hydrophobicity, and buffering capacity play a major role for efficient transport and biological activity of the gene drugs. Insight into the structure–function relationship may form the basis for rationalization of the development of improved gene delivery systems for *in vivo* application.

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10 Subcellular Fate of Proteins and Nucleic Acids

David Oupický

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10.1 INTRODUCTION

Improved understanding of the molecular mechanisms of diseases results in the rational development of drugs, which are designed to act at specific sites within cells. Irrespective of its size, such a drug must safely reach not only its target cell but also the appropriate location within the target cell to achieve the desired therapeutic effect. In the simplest scenario, small molecule drugs enter cells by diffusion and reaching the desired subcellular location is comparatively unproblematic. Unlike small molecule drugs, macromolecular drugs such as proteins, nucleic acids, and synthetic polymer-drug conjugates are restricted to fewer subcellular compartments due to their large size. Macromolecules cannot cross cell membranes by simple diffusion and are usually internalized by endocytosis. The limited ability of macromolecules to enter cells is widely exploited to their advantage by attaching ligands to macromolecules that can target them to specific cells; capitalizing on the unique expression of some plasma membrane receptors and antigens in certain cells or diseases. However, even after the internalization by endocytosis, the macromolecules remain separated from the intracellular space by a biological membrane. Efficient delivery of therapeutic macromolecules to designated subcellular locations therefore greatly relies upon successful passage across biological membranes. For example, delivery of macromolecules to the most important subcellular target, which is the nucleus, requires crossing two biological membranes. Improved understanding of differences in membrane function, properties, and structure among cellular organelles contributes to better control of subcellular delivery and targeting of drugs and becomes increasingly important for the development of macromolecular therapeutics.

This chapter discusses general aspects of subcellular trafficking of macromolecules and polymeric particulate systems, while emphasizing their relevance for the development of macromolecular therapeutics with controlled subcellular trafficking. Current understanding of subcellular trafficking and methods how to overcome the major barriers for cellular delivery of macromolecules are examined for three main classes of polymer-based delivery systems: polymer conjugates, nanoparticles and microparticles, and self-assembly complexes of nucleic acids. The main focus of the chapter is on how the delivery systems can affect intracellular fate of therapeutic nucleic acids and proteins. In each category, the advantages and disadvantages of the respective delivery systems for subcellular trafficking are discussed.

10.2 SUBCELLULAR TRAFFICKING OF MACROMOLECULES

Macromolecules and particles are generally impermeable through cell membranes and cannot enter cells by passive diffusion across the plasma membrane. It has been well established that macromolecules and particles enter cells via endocytosis. In fact, fluorescently labeled macromolecules such as dextrans are often used as markers of endocytosis in studies of subcellular trafficking. This section provides an overview of the endocytosis, necessary for better understanding of subcellular behavior of the macromolecular and particulate delivery systems discussed later. We also briefly examine cytoplasmic and nuclear trafficking of macromolecules, and discuss the possibility of using other subcellular vacuoles as therapeutic targets for macromolecular drugs. Further, more detailed, discussions of these topics can be found in modern textbooks of cell and molecular biology, monographs, and review journals.^{1–8}

Endocytosis is a multi-step process of cell surface invagination and subsequent internalization of the plasma membrane as vacuoles for the cellular uptake of extracellular macromolecules and ligands (Figure 10.1). Macromolecules containing ligands that recognize specific receptors on the cell surface are internalized by receptor-mediated endocytosis. Although some cell receptors may be able to facilitate uptake into the cytoplasm directly across the plasma membrane, the most common route for receptor-mediated uptake of macromolecules is the endolysosomal trafficking pathway. In contrast, macromolecules that are not able to recognize any specific



FIGURE 10.1 Schematic representation of endocytosis and nuclear transport. (Adapted from Varga, C. M. et al., *Biotechnol. Bioeng.*, 2000; 70: 593, and Munkonge, F. M. et al., *Adv. Drug Deliv. Rev.*, 2003; 55: 749.)

receptors are taken up by a slower fluid phase endocytosis (also called pinocytosis)-a nonspecific process for transporting macromolecules dissolved in the bulk extracellular fluid to the cells. The first step in the receptormediated endocytosis is binding of the ligand to its receptor on the cell surface. Ligand binding usually triggers localization of receptors into pits that are coated on their cytoplasmic side of the membrane with the protein clathrin. Approximately 1000 coated pits with a typical diameter of 100 nm can be found on the cell surface at any given time. Coated pits invaginate and constrict to form vesicles called early endosomes. Within 10 min after internalization, the clathrin coating is removed from early endosomes by cytoplasmic enzymes and multiple endosomes fuse to form larger sorting endosomes. Trafficking from sorting endosomes may result in delivery of endosomal content back to the cell membrane via recycling endosomes (they bud from the tubular parts of sorting endosomes) or to late endosomes and lysosomes for degradation. Some receptors and ligands are neither recycled nor degraded; instead they are routed to other intracellular compartments such as trans-Golgi network. Immediately after internalization, endosomes undergo continuous acidification from the initial plasma pH (\sim 7.5) to that found in lysosomes (~4) through the action of ATP-dependent proton pumps. The acidification of endosomes can yield the dissociation of ligands from receptors for pH sensitive ligand/receptor interactions. Most receptors, including insulin, asialoglycoprotein, and LDL receptors bind their ligands tightly at neutral pH but release the ligands if the pH is lowered to below 5. Ligands dissociated from receptors in the sorting endosomes partition between recycling and late endosomes based on relative volumes of the two vesicles. The sorting of internalized ligands and receptors is influenced by the physicochemical properties of the ligand/receptor complexes, including association and dissociation kinetic rate constants and ligand valency. Endosomal sorting is a critical step in the endocytic pathway, because at this point the cell determines the destinations of the endocytosed molecules. Lysosomal enzymecontaining vesicles bud from the trans-Golgi network and fuse with late endosomes, causing late endosome maturation into lysosomes. This results in degradation of contents by lysosomal hydrolytic enzymes. The directed membrane trafficking of endosomal vesicles inside the cells is primarily driven by molecular motors tracking along microtubules and microfilaments. Endocytosis operates also for more complex receptor binding species including many viruses that use endocytic pathway to penetrate cells.

Since macromolecules internalized by both receptor-mediated and fluidphase endocytosis are eventually transferred to lysosomes for degradation, endocytosis represents the ideal pathway for targeting therapeutics for treatment of diseases and disorders of lysosomes (lysosomal infections, metabolic disorders). Endolysosomal trafficking pathway also plays an important role in antigen delivery and processing and therefore its understanding is vital for various immunotherapy strategies. It is well established that major histocompatibility complex class II (MHC II) molecules present antigen derived from endocytic compartments. In contrast, MHC class I molecules present antigen typically generated via the cytosolic pathway of antigen processing and presentation, and thus primarily those derived from endogenous proteins. Such type of adaptive immunity is important and often essential in the protection and clearance of viral infections and tumors. Immunizations with exogenous vaccine antigen fail in most cases to induce strong responses because of the limited cytoplasmic accessibility to gain efficient access to cytosolic antigen processing and presentation. Antigen delivery strategies are important aspects of vaccine development that can benefit from enhanced ability to control subcellular trafficking of macromolecules.

Macromolecules that must reach subcellular targets other than endolysosomes for their therapeutic effect, must escape from endosomes to cytoplasm before lysosomal degradation. Several potential target destinations exist for macromolecular drug succeeding in escaping from endosomal trafficking pathway into the cytoplasm (by fusing with endosomal membrane or by disrupting the membrane). For many macromolecular drugs, such as antisense oligonucleotides, the cytoplasm itself is the final destination. The fluid region of the cell cytoplasm called cytosol (pH \sim 7) contains a cytoskeleton composed of at least three classes of fibers that help to maintain cell shape and mobility and provide anchoring points for other subcellular structures. The cytosol is also a major site of cellular metabolism and contains

large number of different enzymes. In fact, about 20-30% of the cytosol is protein and the cytosol contains 25-50% of the total protein within cells. Although small molecules can diffuse freely and rapidly in the cytoplasm, passive diffusion of macromolecules is often severely limited.⁹ The viscosity of the cytosolic fluid is only 1.2 to 1.4 times greater than the viscosity of water.^{10–} ¹² The translational diffusion of macromolecules with molecular weight less than 500 kDa in cytoplasm is only 3 to 4-fold slower than in water but is markedly slowed for larger macromolecules. The diffusion does not generally depend greatly on the size of macromolecule up to at least a 30 nm gyration radius, but is dramatically slowed for larger macromolecules. The principal barrier for diffusion of macromolecules are collisional interactions due to macromolecular crowding.¹³ Rapid cytosolic transport of macromolecules therefore cannot rely on passive diffusion. Instead, rapid and directional cytosolic transport of macromolecules and subcellular organelles proceeds along microtubules with the help of kinesin or dvnein motor proteins. Microtubules are fibers (24 nm in diameter) that are part of the cytoskeleton. They are formed by polymerization of α,β -tubulin monomers. Vesicles and protein particles often are transported along microtubules in a process mediated by kinesin or dynein.

Nucleus contains chromatin and the machinery necessary for gene transcription separated from the cytoplasm by nuclear envelope. The nucleus is spatially organized into several distinct domains bound by the doublemembrane nuclear envelope. Within the nucleus are the nucleoli, nuclear lamina, and possibly specialized domains for the localization of replication, transcription, and splicing. Nucleus is by far the most important subcellular target of drug delivery strategies. Although access to the nucleus is highly restricted for large molecules, number of macromolecules must enter and exit the nucleus while performing the basic cellular functions. Proteins like transcription factors, for example, are synthesized in the cytoplasm but must be transported to their site of action in the nucleus. It has been established that the bidirectional macromolecular transport to the nucleus proceeds through channels in the nuclear envelope called nuclear pore complexes (NPCs). The NPC (~125 MDa) is built of a set of unique proteins known collectively as nucleoporins and associated nuclear and cytoplasmic filaments surrounding the central channel of NPC (Figure 10.1). The NPC allows rapid transport of small molecules up to 9 nm (~ proteins up to about 50 kDa) by passive diffusion. The diameter of the central channel in NPC can expand from 9 nm up to about 28 nm¹⁴ and permit active transport of larger molecules. A typical cell nucleus contains about 2000 NPCs (i.e., 4 NPCs/mm²)¹⁵ and each NPC can carry out up to 1000 transport events per second.¹⁶ Active nuclear import of proteins larger than 50 kDa is mediated by nuclear localizing signals (NLS). NLS in the best described pathway is recognized by a heterodimeric protein complex of importin- α and importin- β . Importin- α interacts with the NLS sequence while importin- β docks, with the help of the filaments, the complex to the NPC which initiates translocation of the NLS cargo across the NPC (Figure 10.1).

One of important emerging targets for subcellular drug delivery strategies is mitochondria. Most human cells contain substantial number of mitochondria in their cytoplasm whose principal function is ATP synthesis by oxidative phosphorylation. Mitochondria supply most of our ATP and consume nearly all of the oxygen we breathe. The majority of mitochondrial proteins is encoded by nuclear DNA, translated on cytoplasmic ribosomes, imported into mitochondria in unfolded polypeptide state and there folded into functional proteins. Mitochondria also contain their own DNA, which in mammals encodes 13 polypetides, along with 22 tRNA genes, and two rRNA genes necessary for their translation. Mitochondrial DNA diseases arise due to mutations, deletions, or insertions to mitochondrial DNA, which often lead to dysfunctional ATP synthesis and a range of progressive neuromuscular disorders, obesity, diabetes, and cancer. There is currently no curative treatment for mitochondrial dysfunctions. To deliver macromolecules to mitochondria, they must first cross the plasma membrane into the cytoplasm where they should be stable and remain in an import competent form and then targeted selectively to mitochondria using specific peptide targeting ligands.^{17–25}

10.3 EXPERIMENTAL METHODS

In order to elucidate subcellular trafficking of macromolecular therapeutics, we must be able to follow macromolecules temporally and spatially throughout the cell. Unfortunately, synthetic and natural macromolecules can only rarely be visualized within cells without the use of additional agents. The most popular experimental methods for monitoring the subcellular fate involve following macromolecules labeled with radioactive, fluorescent, and electron-dense markers. A typical experiment involves incubation of cells with labeled macromolecules for a specified time and observing the cellular localization of the labels either in chemically fixed or live cells. Fluorescence microscopy techniques are the main tool used to follow the trafficking of labeled macromolecules in the intact live cells. A particular advantage offered by fluorescent labels is the ability to discriminate between the therapeutic and carrier macromolecules when separate labels with unique spectral properties are used for the two macromolecules. Commonly used fluorophores that allow such discrimination include fluorescein and rhodamine. Proteins, nucleic acids, and synthetic macromolecules can be easily labeled with fluorescent labels using a variety of commercially available reagents. If fluorescence intensity of label varies with pH, such as in case of fluorescein, it can be used to follow changes of pH during the movement within the cell.^{26,27} Fluorimetric methods, such as fluorescence flow cytometry can be used to measure the amount of fluorescently-labeled systems internalized by the cells. Disadvantages to the use of fluorescence labels occur when high light intensities are needed to produce a strong fluorescence signal, since the fluorophore may photobleach. Another disadvantage of fluorescent labels is a limited possibility to obtain quantitative information about the

subcellular trafficking and the possibility that attachment of a large fluorescence label moiety can significantly alter the properties of the studied macromolecules. Radiolabeled macromolecules, on the other hand, are used for quantitative determination of the subcellular trafficking. To quantify amounts of internalized macromolecules in specific intracellular vacuoles, one must first physically separate the organelles, usually on the basis of their differential density using gradient sedimentation. This enables quantification of the amount of labeled species in a variety of organelles, including endosomes, lysosomes, mitochondria, and nuclei. Such quantitative studies are nevertheless rather difficult experimentally because the species must be followed as they move through various cellular organelles. The most common radioisotopes used for radiolabeling of macromolecules include iodide (¹²⁵I), phosphorus (³²P), and sulfur (³⁵S). Lastly, the subcellular localization of macromolecules can be visualized by electron microscopy using electron-dense labels such as colloidal gold or osmium tetroxide. The experimental procedure usually involves incubation of the cells with the macromolecules conjugated with colloidal gold nanoparticles prior to fixation for microscopy. Since colloidal gold is available in a range of specific sizes, simultaneous labeling and thus localization of different macromolecular species is possible.

10.4 SUBCELLULAR FATE OF NUCLEIC ACIDS AND PROTEINS

This section examines the subcellular fate of macromolecular therapeutics from the perspective of the contribution of the used carrier system. Both types of macromolecular drugs discussed here, i.e., proteins and nucleic acids, can be attached to the carrier polymer by covalent bonds, physically entrapped or dispersed within polymer carrier, or bound non-covalently to the carrier molecule. Accordingly, we describe general subcellular trafficking patterns of the three main soluble carrier systems of macromolecular therapeutics: polymer conjugates; nanoparticles and microparticles; and self-assembly systems. Although the macromolecular drugs may have a strong influence on the behavior during cellular delivery, the general aspects of subcellular fate of nucleic acids and proteins are mainly determined by the carrier system used.

10.4.1 POLYMER CONJUGATES

A number of water soluble polymers have been investigated as potential carriers for delivery of drugs, including synthetic polymers such as poly (ethylene glycol) (PEG),^{28–32} *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers,³³ dendrimers,³⁴ polypeptides,^{34,35} as well as natural polymers such as dextran.³⁶ All the mentioned polymers usually share similar subcellular trafficking properties in that they cannot enter cells by diffusion and are internalized by endocytosis or pinocytosis and are ultimately trafficked to lysosomes. Attachment of small molecule drugs or macromolecular drugs to

these polymers typically leads to a substantial modulation of disposition properties and, especially in the case of small molecule drugs, significantly alters the mechanism of cell entry. In general, the subcellular fate of polymer conjugates is often predetermined by that of the polymeric carrier.^{33,37} The most widely investigated polymer conjugates of macromolecular drugs are protein conjugates followed by polymer conjugates of antisense oligonucleotides.^{28,38,39}

Despite intensive research of polymer conjugates, surprisingly little attention has been devoted to understanding the subcellular fate of the protein and nucleic acid conjugates so far. HPMA copolymers and their conjugates with drugs are among the most extensively studied in the field of macromolecular therapeutics. Subcellular trafficking of HPMA copolymers and their conjugates with proteins, peptides, and antisense oligonucleotides has been investigated comparatively to a greater detail than that of conjugates of other polymers.^{33,40–43} This section therefore focuses on the description of subcellular behavior of HPMA copolymers and their conjugates, but many of the general features appear to be applicable also to other polymer conjugates. HPMA copolymers have been studied for drug delivery and other biomedical applications for decades. HPMA homopolymers are hydrophilic and typically exhibit a low affinity for cell membranes. The chemical properties of HPMA allow easy preparation of linear copolymers and incorporation of a variety of functional groups that can be utilized for attachment of targeting ligands, small molecule drugs, and macromolecular drugs. Biodegradable as well as non-biodegradable spacers can be utilized to connect the drug molecules to the polymer backbone (Figure 10.2). Tetrapeptide Gly-Phe-Leu-Gly (GFLG) is an example of biodegradable spacers.^{44,45} GFLG spacer is stable in the extracellular space but is efficiently cleaved by proteolytic enzymes in the lysosomes, which leads to the release of the attached drug molecule. Conjugation via the GFLG spacer also prevents release of drugs before reaching the lysosome of cells. In contrast, dipeptide glycylglycine is a typical example of a nondegradable spacer.⁴⁶ HPMA copolymers are biocompatible but not biodegradable and their molecular weight must be tailored so that easy elimination from the organism by renal filtration is ensured.^{47,48} The typical molecular weight of HPMA copolymers used in drug delivery strategies is \sim 30,000.

HPMA copolymers containing various fluorescent labels (typically 1 to 3 molecules of the label per macromolecule) or radioactive labels (¹²⁵I) are the usual tool for studying subcellular trafficking of HPMA conjugates. The hydrophilic character and large size of HPMA copolymers and their conjugates prevents them from easy entering as many cellular compartments as small molecules that enter cells by diffusion. HPMA copolymers alone are typically taken up by cells by the relatively slow process of fluid phase endocytosis (pinocytosis). Adding targeting ligands to the copolymers allows their targeting to specific cells and results in the increased total levels and rate of cellular uptake. Lysosomal delivery is the most common fate of endocytosed material and HPMA copolymers are no exception. Shortly after

incubation with cells, the HPMA copolymers are usually localized in endosomes and lysosomes, where, being resistant to degradation, HPMA copolymers accumulate. Drugs sensitive to lysosomal enzymes are quickly degraded after localization to lysosomes if additional steps are not taken to protect them or to facilitate their escape into the cytoplasm. The known properties of lysosomal membrane that does not allow easy diffusion of even small molecules⁴⁹ seem to prevent HPMA copolymers from escaping into the cytoplasm without the use of auxiliary agents to enhance the penetration. However, HPMA copolymers can enter into the cytoplasm^{50,51} and nucleus⁵¹ of at least several cell lines including human hepatocarcinoma HepG2 cells without the use of any membrane disruptor, pH stabilizer, or any other chemical or physical intervention. The cytoplasmic escape is a relatively slow process, requiring at least 3 h to observe its first evidence and it typically takes more than 24 h to see more than 25% of the cells with copolymers localized in the cytoplasm. After endosomal escape into the cytoplasm, HPMA copolymers rapidly, within minutes, accumulate into the nucleus. The lysosomal escape and nuclear accumulation of hydrophilic HPMA copolymers that have almost no net charge cannot be explained on the basis of current knowledge of subcellular trafficking of macromolecules and additional studies are required to fully understand the cellular processes involved. It also remains unknown how much of the endocytosed copolymers is able to escape into the cytoplasm and partition to the nucleus.⁵²

Attachment of protein molecules such as transferrin or monoclonal antibodies to HPMA copolymers does not significantly alter the general pattern of subcellular trafficking.^{53,54} The conjugates are usually taken up rapidly by the cells by receptor-mediated endocytosis if corresponding receptor is present and are routed via endosomes into the lysosomal compartment, where the protein is degraded while nonbiodegradable HPMA copolymer remains intact. Conjugation of antibodies to HPMA copolymer increases markedly lysosomal accumulation of the copolymer. Rapid lysosomal degradation of the attached protein molecules prevents the conjugates to escape into the cytoplasm or nucleus.

HPMA conjugates with antisense oligonucleotides (ODNs) confirm the common features of the subcellular trafficking described for HPMA copolymers alone and their protein conjugates.^{39,52} The basic features of subcellular trafficking were established by investigating the fate of fluorescently labeled HPMA copolymer containing fluorescently labeled ODNS attached via biodegradable GFLG or nonbiodegradable GG spacers (Figure 10.2). The use of two different fluorescent labels allows one to discriminate between trafficking of ODNs and HPMA copolymers during their cellular processing. Conjugating the ODNS to the HPMA copolymers directs their delivery into endolysosomal pathway, ultimately leading to lysosomal localization. The lysosomal destination of ODNs that must bind to specific target mRNA in the cytoplasm or nucleus to exert their therapeutic effect represents a significant disadvantage of such polymer conjugates. Free ODNs can enter some cells, such as HepG2 cells, via receptor-mediated endocytosis⁵⁵ but also



FIGURE 10.2 Schematic representation of HPMA copolymer conjugates with antisense oligonucleotides.

accumulate in vesicles, probably endosomes and lysosomes, in the cytoplasm. ODN-HPMA conjugates containing the GG spacer are taken up by the cells by endocytosis and vast majority of HPMA copolymers and ODNs remains in the lysosomes with no significant changes over time because of the used nondegradable spacer. Only a very small amount of the conjugates escapes to the cytoplasm. While binding ODNs to the HPMA copolymers via nondegradable GG spacer prevents ODNs from entering the cytoplasm and nucleus, conjugating via the lysosomally degradable GFLG spacer results in significantly altered subcellular distribution. Initially, both ODNs and HPMA copolymer are found together in small cytoplasmic vesicles, probably endosomes and lysosomes. Later, the two components separate as ODNs are detached from the HPMA copolymer and translocate to the cytoplasm and the nucleus; similarly to the fate of free ODNs. ODNs conjugated to the HPMA copolymer by the degradable GFLG spacer are released from the polymer and escape from lysosomes in a fashion similar to the free ODNs. The ODNs escape from lysosomes faster that the HPMA copolymer itself. The HPMA copolymer increases the activity of ODNs by increasing its stability in the lysosomes. The presence of ODNs also changes the properties of HPMA copolymers that prevent the HPMA copolymers from accumulating in the nucleus as observed for free copolymers.⁵¹

10.4.2 NANO-/MICROPARTICLES

Polymeric nanoparticles and microparticles are used for delivery of small molecule drugs as well as macromolecular drugs such as proteins and nucleic acids (Chapter 14).^{56–61} In comparison to small molecule drugs, encapsulating large hydrophilic molecules like proteins and DNA in relatively small hydrophobic particles represents unique formulation challenges. Nevertheless,

the subcellular behavior of polymeric particles appears to be primarily influenced by the used polymer rather than the encapsulated drug. This section provides an overview of the current understanding of the cellular uptake and trafficking of polymeric nanoparticles and microparticles.

Both nanoparticles and microparticles have been shown to enhance the delivery of a variety of drugs across natural membranes. However, the smaller size of nanoparticles offers several distinct advantages because of the way size of particles influences their interactions with cells, mode of cell internalization, and to certain extent also subcellular trafficking. Additionally, systemic disposition profile of particles also depends on the size, and so nanoparticles can usually penetrate through smaller capillaries than microparticles, which enables an efficient drug accumulation at the target sites. One of the most important characteristics determining the rates and total levels of cellular uptake of particles is their size.^{62,63} The size-dependency of cellular uptake was demonstrated on particles made of various polymers. Fluorescent-labeled polystyrene nanoparticles of 20 nm size, for example, are internalized avidly by a variety of cell lines, while cellular uptake of larger polystyrene particles depends on cell type. Whereas some cell lines take up particles up to 1 µm, others are not capable of internalizing particles larger than ~ 100 nm.⁶³ Similar behavior was observed for cellular uptake of biodegradable polv(D.L-lactide-co-glycoside) (PLGA) particles of mean diameters 100 nm, 1 µm, and 10 µm containing bovine serum albumin as a model protein and a fluorescent marker.⁶⁴ The 100 nm size PLGA nanoparticles exhibit 2.5-fold greater uptake on the weight basis than 1 µm and six-fold higher uptake compared to 10 µm PLGA microparticles. In terms of particle numbers, the uptake of 100 nm particles is 2700-fold greater than the 1 µm and 6.7×10^6 greater than the 10 µm diameter microparticles. The efficiency of uptake is also significantly greater for small particles; 41% for 100 nm nanoparticles compared with 6% for the 10 µm microparticles was observed in the studies.⁶⁴

The mechanism of cellular uptake and subcellular behavior were studied in detail for PLGA nanoparticles.^{65,66} PLGA nanoparticles are internalized by cells via a saturable endocytic process. Existing evidence suggests that the internalization involves in part fluid-phase endocytosis and in part clathrin-coated pits. Involvement of other uptake processes such as phagocytosis has not been observed for PLGA nanoparticles. Shortly after cellular uptake, the nanoparticles are transported to primary endosomes and then probably to sorting endosomes. From sorting endosomes, a fraction of nanoparticles is recycled back to the cell surface through recycling endosomes, while the remaining fraction is transported to late endosomes and lysosomes. Within 10 min of cellular uptake, some PLGA nanoparticles escape the endolysosomal pathway and enter into the cytoplasm (Figure 10.3). The escape into the cytoplasm is attributed to the cationization of PLGA nanoparticles in the acidic environment of endosomes and lysosomes. The cationization of PLGA nanoparticles with decreasing pH is caused by the transfer of excess protons from the solution to the nanoparticles surface or by



FIGURE 10.3 Subcellular fate of poly(D,L-lactide-*co*-glycoside) (PLGA) nanoparticles observed by transmission electron microscopy. Osmium tetroxide-loaded nanoparticles (indicated by arrows) are observed in the cytoplasm of human vascular smooth muscle cells (A), and interacting with vesicular/endosomal membrane (B). Bars represent 500 nm. (From Panyam, J. et al., *Int. J. Pharm.*, 2003; **262:** 1, with permission.)

hydrogen bonding between carboxyl acid groups of PLGA and hydronium cations in the acidic pH. It is assumed that PLGA nanoparticles exhibit charge reversal from the original negative values at the physiological pH (7.5) to a net positive charge in the acidic vesicles. The positively charged nanoparticles can electrostatically interact with the endosomal and lysosomal membranes (Figure 10.3). Such interaction, which does not cause disruption of the endosomes or lysosomes, then probably leads to a localized destabilization of the endosomal membrane and the escape of the nanoparticles into cytoplasm. The cytoplasmic transport is unique to PLGA nanoparticles, as nanoparticles based on other polymers, which do not show a similar pH-dependent changes of surface charge, are retained in the endosomes.⁶⁷ The cytoplasmic delivery makes PLGA nanoparticles well suited for sustained cytoplasmic release of encapsulated drugs whose targets are cytoplasmic. Efficient cellular uptake of PLGA nanoparticles is dependent on maintaining sufficient concentration gradient between intracellular and extracellular space. When the nanoparticles are removed from external medium, exocytosis of nanoparticles results in a rapid decrease of intracellular levels. Additional factor influencing cellular uptake of PLGA nanoparticles is the presence of residual emulsifiers such as polyvinyl alcohol. The presence of polyvinyl alcohol in the PLGA nanoparticles affects their surface properties and leads to decreased cellular uptake.⁶⁸

It was already mentioned that microparticles are generally internalized by cells to a significantly lesser extent than nanoparticles. This does not prevent using microparticles as drug depots exhibiting prolonged release kinetics and long persistence at the site in applications that do not require cellular uptake of the particles. Importantly, however, microparticles represent an attractive delivery system to target professional antigen presenting cells (APCs) for the delivery of protein and DNA vaccines. 59,60,69-79 As vaccine targets, macrophages and dendritic cells (DCs) are the most important types of professional APCs. Many of APCs have the unique capability to efficiently internalize foreign particulate material such as microparticles, bacteria, and dust particles. The APCs take up microparticles by a process known as phagocytosis (Figure 10.4), which results in creating an intracellular organelle called phagosome. Phagosomes usually fuse with lysosomes, creating phagolysosomes where the ingested material is degraded. In contrast to endocytic uptake of nanoparticles, phagocytosis of larger microparticles is usually more efficient than phagocytosis of smaller ones.⁷⁸ The efficiency of the cellular uptake also depends on the surface charge of microparticles, with negatively charged microparticles exhibiting stronger size-dependence than positively charged ones. It has been established that macrophages that take up microparticles deliver them within 15 min into the lysosomes, irrespective of the chemical nature of polymer used.⁷¹ However, the nature of the polymer used affects postlysosomal fate of the delivered antigens. Microparticle-associated antigens typically exhibit prolonged intracellular retention, which affects the ability to deliver macromolecules to MHC I and II antigen presentation pathways. As already mentioned, MHC II molecules present antigen derived from endocytic compartments while MHC class I molecules present antigen derived from the cytoplasm. It has been



FIGURE 10.4 (See color insert following page 512) Phagocytosis of fluorescent-labeled polystyrene microparticles ($4.5 \,\mu$ m) by dendritic cells imaged by confocal laser scanning microscopy. Albumin-coated microparticles attached to the surface of dendritic cells are shown in (a) and internalized microparticles by dendritic cells are shown in (b). Bars represent 10 μ m. (From Thiele, L. et al., *J. Controlled Release*, 2001, **76**: 59, with permission.)

established that biodegradable microparticles can deliver antigens directly from phagosomes into cytoplasm and permit the antigens to follow the conventional route for MHC I presentation.^{70,79–81} The results show that antigens associated, for example, with polystyrene and poly-*\varepsilon*-caprolactone (PCL) microparticles are delivered from phagosomes into the cytosolic space of macrophages. It is speculated that interaction of the solid microparticles with phagosomal membrane facilitates its disruption and leads to cytoplasmic delivery. Once delivered into the cytoplasm, the antigens are processed in proteasomes and transported via the Golgi for MHC I presentation. Efficient cytoplasmic delivery is enhanced by the use of biodegradable microparticles, such as those based on PCL.⁷⁰ Despite lower phagocytic activity compared with macrophages, dendritic cells are an attractive target for microparticle-mediated antigen delivery because they represent the most potent APCs. They are capable of stimulating MHC I restricted CD8+ cytotoxic T-lymphocytes that are important in fighting viral infections and tumor cells. Similar to macrophages, significantly improved antigen presentation is observed when the antigen is contained within microparticles and delivered via phagocytosis compared to the fluid-phase uptake of soluble antigens.⁷⁴

10.4.3 POLYPLEXES

Polyplexes are polyelectrolyte complexes formed by self-assembly of nucleic acids with polycations (Figure 10.5). The term polyplexes is generic for polycation complexes of high and low molecular weight DNA and RNA that are utilized in various gene therapy protocols (see Chapter 18 for detailed description of the various nucleic acids). Typical polyplexes used in gene delivery are formed with excess polycation to efficiently condense the nucleic acid and provide protection against enzymatic hydrolysis. As a result, polyplexes are highly positively charged nanoparticles with rather wide distribution of sizes in the range of 25 to 500 nm and often exhibit low colloidal stability. Delivery properties of polyplexes are primarily controlled by the used polycation. Factors such as molecular weight, chemical structure, charge density, buffering capability, and polyplex stoichiometry are among the most influential. Importantly, nucleotide sequence of the used nucleic acid does not affect the delivery properties of polyplexes. Subcellular trafficking of polyplexes has been investigated comparatively more than similar behavior of polymer conjugates and nanoparticles. Current level of understanding of subcellular trafficking of polyplexes collectively suggests that the efficiency of the process can be controlled by the physicochemical properties of the vectors. Nevertheless, because of the complexity of the vectors themselves and that of the delivery process more information is still needed to fully understand the processes involved. Overall, the intracellular transport of polyplexes is still poorly understood, limiting the rational design of efficient new vectors.^{82,83}



FIGURE 10.5 Schematic representation of the subcellular trafficking of polyplexes. The polyplexes are formed by self-assembly of plasmid DNA and multiple polycation molecules and internalized by the cell by endocytosis. Some polyplexes escape from the endosomes into the cytoplasm, while the rest is routed for degradation into lysosomes. Polyplexes located in the cytoplasm undergo full or partial disassembly or are translocated, probably along microtubules, to the perinuclear region. DNA from the polyplexes that undergo cytoplasmic disassembly can be degraded by cytosolic nucleases or transported to the nucleus. Polyplexes are transported into the nucleus either passively by association with nuclear material during breakdown of the nuclear envelope during cell division or actively through nuclear pore complexes. Once inside the nucleus, polyplexes disassemble and allow the transcription apparatus access to the DNA.

It is estimated that about one million DNA molecules needs to be delivered by a polyplex vector to a single cell to transfect it. In comparison, a single virus particle can, in principle, achieve transfection of a cell. Such a low efficiency of polyplex gene delivery is a consequence of limited capability of polyplexes to deal with the numerous barriers on the way to the subcellular site of action. Although localization of therapeutic DNA to cell nucleus is
necessary for gene expression to take place, various other therapeutic nucleic acids such as RNAs function in different compartments, and their appropriate localization is equally necessary for their activity. In the following discussion, we follow subcellular pathway of plasmid DNA polyplexes from the point of the association with plasma membrane to their arrival at the cell nucleus (Figure 10.5).

Although DNA is easily internalized by certain liver macrophages via anionic scavenger receptors,^{84–87} most cells lack such capability. Cellular association of DNA is therefore usually very low due to high negative charge density that prevents binding to negatively charged plasma membrane. Positively charged polyplexes provide a reliable way to substantially increase the cellular association of DNA. In the absence of specific targeting ligands capable of inducing receptor-mediated endocytic internalization, polyplexes are taken up by the adsorptive endocytosis. They bind to the negatively charged heparan sulfate proteoglycans located on the cell surface.^{88,89} These proteoglycans are present either as an integral part of cell membrane or as extracellular proteins closely associated with cell surfaces. Adsorptive endocytosis most likely involves direct nonspecific binding to the plasma membrane proteoglycans. Attachment of protein-, peptide-, and carbohydratebased targeting ligands can lead to cell entry via receptor-specific endocytosis and as such avoid unwanted effects in non-target cells. Polyplexes targeted with galactose,⁸⁴ transferrin,⁹⁰ epidermal growth factor,⁹⁰ folate,^{91,92} anti-bodies and their fragments,^{93,94} and integrin-binding peptides^{95–97} were used for cell-specific delivery of genes. Adsorptive and receptor-mediated endocytosis are more efficient and faster processes than fluid phase endocytosis and phagocytosis that can potentially be also involved in the internalization of polyplexes. After binding, the endocytosis pathway of polyplexes is largely assumed to mirror that of regular ligand/receptor internalization (Figure 10.1).^{98,99} Polyplex binding to a cell surface triggers aggregation into coated and noncoated pits and internalization into endosomes. The major difference between polyplexes and individual ligands in their endosomal behavior is the multivalent character of polyplex binding that substantially increases their avidity. Ultimately, however, fusion with lysosome enriched with hydrolytic enzymes yields the digestive vacuoles and the DNA is subjected to degradation.¹⁰⁰⁻¹⁰²

In order to avoid the hydrolysis of delivered DNA in the lysosomes, polyplexes must find their way out of the endolysosomal pathway. Despite the high positive surface charge that favors intimate contact with endosomal membrane, not all polyplexes have substantial capability to cross endosomal membrane. The ability to escape the endolysosomal pathway depends primarily on the chemical structure of the polycation used. A class of pH-sensitive polycations represented by polyethylenimine (PEI),^{103,104} poly-amidoamine dendrimers,¹⁰⁵ and histidylated poly(L-lysine)¹⁰⁶ represents polycations that exhibit sufficient capability to facilitate endosomal escape and efficiently transfect a variety of cells. PEI in particular, is among the most efficient nonviral agents for in vitro and *in vivo* gene transfer.¹⁰⁷

The mechanisms involved in endosomal release of PEI-based polyplexes are not yet fully understood, but has been suggested to be the "proton sponge" hypothesis.¹⁰⁸ The hypothesis is based on the chemical structure of the PEI; an organic polymer with high cationic-charge density and considerable buffer capacity over a wide range of pH. Endosomal membrane contains a proton pump that acidifies the compartment. The proton flux into endosomes containing PEI polyplexes causes protonation of PEI, which provokes a massive proton accumulation followed by passive influx of water and chloride ions. These events cause osmotic swelling and subsequent endosome disruption, thus permitting the escape of endocytosed polyplexes into cytoplasm. The hypothesis is supported by slowed endosomal acidification, increased endosomal volume, endosomolysis, and dependence of transfection activity on endosomal acidification observed for PEI polyplexes.^{27,109,110} Additional direct support for the proton sponge hypothesis was provided when the burst of intracellular organelles and release of PEI polyplexes into cytosol have been observed by live cell confocal microscopy.¹¹¹ Additionally, size of PEI polyplexes is another factor apparently affecting the endosomal escape. Larger polyplexes can reduce the rate of the transfer of endocytosed DNA to lysosomes, which allows DNA to reside much longer in endocytic compartment, thus protected from lysosomal nucleases and available for transfer to cytoplasm.¹⁰² Larger PEI polyplexes also seem to mediate more pronounced "proton sponge" effect due to higher amount of PEI in the endosomes.¹¹² It is noteworthy, however, that buffering capability itself is not sufficient to make a polycation a good carrier for DNA delivery, as documented by various polycations exhibiting high buffering capability but without the corresponding transfection activity.^{105,113,114} Indirect evidence suggests that cationic polymers must also possess a highly flexible structure to be a good DNA carrier.^{105,114} The efficiency of gene transfer of less active polyplexes can be enhanced by incorporating membrane-destabilizing peptides derived from viral proteins.¹¹⁵ Additional pharmacological agents such as lysosomotropic drug chloroquine can be also used in combination with low-efficiency polyplexes to enhance endosomal release of DNA. Chloroquine is a hydrophobic weak base that inhibits the transfer of endocytosed material from endosomes to lysosomes.

Current evidence supports a model in which polyplexes released from endolysosomal pathway remain fully or partially assembled in the cytoplasm.^{116,117} Translocation of polyplexes or DNA across cytoplasm is considerably limited due to diffusional barrier and the presence of cytosolic nucleases.^{9,118} Short DNA fragments of ~100 bp diffuse freely in the cytoplasm with a diffusive rate only ~5 times slower than in water and rapidly accumulate in the nucleus. On the other hand, DNA fragments with sizes corresponding to typical plasmid DNA vectors show little or no diffusion. A dramatic reduction of DNA diffusion mobility in cytoplasm occurs when DNA size is increased above 1000 bp. The diffusion of DNA with sizes beyond 3000 bp is immeasurably slow.⁹ Molecular crowding is believed to be primarily responsible for the reduced DNA diffusion, because cytoplasm represents rather crowded cellular environment with 10-15% of volume occupied by macromolecules.¹¹⁹ Overall, cytoplasmic diffusion of DNA is a significant rate-limiting barrier in the nuclear transport particularly in the presence of cytosolic nucleases. Extremely slow passive diffusion of DNA and probably also polyplexes in the cytoplasm suggests involvement of an alternative active mechanism of translocation to nuclear envelope such as involvement of microtubules that account for efficient nuclear delivery of some viruses.¹²⁰ Tracking subcellular movement reveals that transport of individual PEI polyplexes falls within one of three distinct subclasses: diffusive transport, sub-diffusive transport, and active transport ($\sim 20\%$ of polyplexes).¹²¹ Polyplexes exhibiting diffusive transport undergo random, thermally driven motion. Polyplexes undergoing sub-diffusive transport are either physically attached to an intracellular structure or contained within a viscoelastic environment, such as an endosome or molecularly crowded regions of cytoplasm. Polyplexes undergoing active transport move several orders of magnitude faster than polyplexes undergoing simple diffusive transport. In addition, the motion of actively transported polyplexes proceeds along a path that intersects the cell nucleus suggesting involvement of microtubules that meet at the microtubule-organizing center located adjacent to the cell nucleus. Actively transported polyplexes can travel a distance of 10 µm in a given direction in less than $1 \min (\sim 200 \text{ nm/s})$. In contrast, polyplexes relying on simple passive diffusion travel the same distance of 10 µm in almost 9 h. The average velocity of actively transported polyplexes is on the same order of magnitude as movement along microtubules involving motor proteins such as dyneins and kinesins. It is not yet fully understood whether polyplexes escape endosomes and travel along microtubules like adenovirus or reach the perinuclear region within endosomes transported on microtubules like adeno-associated viruses.

After translocation through the cytoplasm, DNA must be delivered to the nucleus for transcription of the transgene to take place. The transport across the nuclear envelope represents one of the major limitations for efficient gene delivery by polyplexes. The precise mechanism of DNA transport to the nucleus and whether the DNA remains associated with the polycation are still largely unknown (Figure 10.5). It appears that whether polyplexes fully or partially disassemble in the cytoplasm depends strongly on the type and length of the used polycation. On one hand, intact polyplexes are often found in the nucleus, indicating that it is not necessary for the polyplexes to disassemble prior nuclear transport.^{122–124} On the other hand, a comparison of the cytoplasmic fate of polyplexes based on linear and branched PEI using fluorescence resonance energy transfer suggests at least partial disassembly of linear PEI polyplexes in the cytoplasm but no disassembly of branched PEI polyplexes.¹¹⁶ It is assumed that this differential cytoplasmic behavior may contribute to the superior transfection activity of linear PEI polyplexes when compared with branched ones. Two modes of nuclear entry are probably utilized by polyplexes. In the first mechanism, polyplexes enter nucleus by association with nuclear material during breakdown of the nuclear envelope during cell division. This mechanism is vitally dependent on the cell cycle* as it is envisioned that polyplexes accumulated in the perinuclear region of the cell await breakdown of the nuclear envelope for entry.^{125,126}

In the second mechanism of nuclear entry, polyplexes or DNA pass to the nucleus through nuclear pore complexes. Short DNA fragments whose cytosolic movement is not significantly hindered are actively imported into the nucleus through the nuclear pores and typically are found in the nucleus within minutes of entering cytoplasm. Transport of polyplexes and large DNA molecules via nuclear pores has been also implicated in numerous studies. Small poly-L-lysine (PLL)-based polyplexes with carefully controlled sizes compatible with transport through expanded nuclear pores (~28 nm) are capable of delivering the DNA into the nucleus of nondividing cells. Marked decrease in transfection activity observed when the minor ellipsoidal diameter of polyplexes exceeds 25 nm confirms the upper size limit of nuclear uptake through nuclear pore complexes.¹²⁷ The size of typical polyplexes exceeds the upper limit for transport through nuclear pores and it is therefore not surprising that they usually rely on the first mechanism for nuclear entry and their activity is strongly cell cycle-dependent. An exception to the cell-cycle dependent activity appears to be polyplexes based on linear PEI. It has been shown that unlike the branched form of PEI, the linear PEI exhibits DNA transfection only modestly dependent on the cell cycle, suggesting improved nuclear import characteristics even in non-dividing cells.¹²⁸ Reporter gene activity mediated by branched PEI polyplexes is typically several hundred-fold higher in cells in S or G2 phase of cell cycle than in cells in G1 phase. In contrast, only small differences in gene expression are observed between cells transfected in G1 phase and those transfected in S/G2 phases when linear PEI polyplexes are used. In addition, DNA is observed in the nucleus within few hours of transfection with linear PEI polyplexes, whereas branched PEI polyplexes remain mostly associated with cytoplasmic structures.¹²⁹ Further enhancement of DNA transport to the nucleus can be achieved by the use of nuclear localization signals or DNA with sequences facilitating nuclear import.^{130,131} Once inside the nucleus, DNA of all sizes are nearly immobile because of extensive DNA binding to nuclear components, including the positively charged histones.⁹

Full or partial disassembly of the polyplexes in the nucleus so that the transcription apparatus can access the DNA efficiently is the final step of gene delivery. Exchange reactions with chromosomal DNA are the most likely mechanism for nuclear disassembly of polyplexes. However, the rates and location of intracellular disassembly of polyplexes can also influence the level and kinetics of transgene expression in a broader way. If polyplexes undergo disassembly too readily, DNA can be released prematurely in cytosol

^{*}Cell cycle is a sequence of events in which a cell duplicates its chromosomes and divides into two. A typical eukaryotic cell cycle can be divided into four phases: G1 before DNA synthesis; S when DNA replication occurs; G2 after DNA synthesis; and M when cell division occurs. Cells can exit the cell cycle during G1 and remain in the G0 state as nongrowing, nondividing (quiescent) cells.

and cytoplasmic DNA degradation occurs, whereas if polyplex disassembly occurs too slowly or not at all, the DNA may be inaccessible to the nuclear transcriptional machinery.¹³⁰ The disassembly rates of the polyplexes can be controlled by the properties of the used polycations. The type of charged center, charge density, and polymerization degree are the most important polycation properties that determine the disassembly rates. Polycations with primary amino groups, high charge density and high degree of polymerization exhibit the lowest disassembly rates.^{132–134} It has been shown that an optimum intermediate polymer length exists corresponding to an optimal disassembly rate that results in maximum gene transcription.^{135,136} The importance of intracellular disassembly can be well demonstrated on RNA delivery. In contrast to DNA, the subcellular target site of action for RNA is cytoplasm. It is believed that RNA polyplexes has to undergo partial or full disassembly in order to mediate efficient transfection. However, very low transfection activity is observed for RNA polyplexes based on high molecular weight polycations that provide efficient DNA transfection.¹³⁷ Low molecular weight polycations or high molecular weight polycations capable of specific cytoplasmic depolymerization, on the other hand, mediate efficient RNA transfection.^{137,138} Overall, it is not yet fully understood how the rates and location of disassembly of polyplexes affect efficiency of gene delivery. It remains to be elucidated whether or to what extent the disassembly takes place already in the cytoplasm or even in endolysosomal pathway. It is likely, however, that depending on the used polyplexes, a range of disassembly properties exists, as documented on the above described differences between linear and branched forms of PEI.¹¹⁶

Closely related to the polyplex disassembly is the effect of cellular nucleases on the gene delivery process. Cytoplasm and lysosomes contain nucleases capable of hydrolyzing DNA and this nuclease activity is a one of barriers to the successful delivery of foreign genes to mammalian cells. One of the benefits of polyplexes is that DNA associated with polycations is significantly more resistant to enzymatic degradation. Premature, partial or full, disassembly of polyplexes in any subcellular compartment containing active nucleases will unavoidably lead to lower transfection activity^{100,101,118,139–141} In particular, cvtosolic calcium-sensitive nucleases can digest DNA when partially disassembled polyplexes are present. The lysosomal DNase II is an additional barrier to effective polyplex gene delivery as documented by enhanced transfection activity in the presence of specific DNase II inhibitors.¹⁰⁰ The relative contributions of cytosolic and lysosomal DNA hydrolysis to overall nuclease effect on transfection activity of polyplexes are not yet known.

10.5 CONCLUDING REMARKS

Subcellular fate of proteins and nucleic acids depends on their physicochemical properties, structure/backbone and the type of carrier molecules used. Polymeric carriers can be used to enhance the cellular uptake, endosomal release, cytoplasmic stability, intracellular trafficking, and nuclear translocation of protein and nucleic acid drugs. The particle size and surface properties of nano-/microparticles have significant influence on the subcellular fate of these macromolecular drugs.

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11 Stability of Proteins and Nucleic Acids

Zheng-Rong Lu

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11.1 INTRODUCTION

Proteins and nucleic acids are the building blocks of life. With the advance of modern science, the functions of these building blocks have been learned and understood more and more. As a result, proteins and nucleic acids including DNA and RNA have been used as therapeutics in modern medicine to treat human diseases caused by the malfunction of these building blocks. However, effectiveness of the new therapeutics is affected by many factors. One of the factors is the instability of proteins and nucleic acids. It has been a challenge for biotechnological and pharmaceutical industries to develop stable formulations of protein and gene therapeutics. In order to stabilize the proteins and nucleic acids in formulations, we need to understand the mechanisms of instability and causes of degradation. Degradation can be prevented or slowed down by avoiding the factors that cause degradation in formulations.

Proteins and nucleic acids are relatively large molecules. Their functions rely on the three-dimensional structures of proteins and the information recorded in the sequence of nucleic acids, respectively. Any damage to the structure will significantly reduce their activity and therapeutic effectiveness. Degradation of proteins and nucleic acids is caused not only by chemical reactions but also physical interactions. The mechanisms of degradation of proteins and nucleic acids and factors that affect their stability have been investigated by scientists in both academia and industries. This chapter summarizes and discusses the major degradation pathways and the possible mechanisms influencing the stability of proteins and nucleic acids.

11.2 STABILITY OF PROTEINS

To understand the stability of proteins, we first need to know the structure characteristics of proteins. Any change of their structures may lead to the loss of their therapeutic functions or may cause toxic side effects. The structure and function correlation of protein therapeutics is much more complicated than that of chemotherapeutics. The therapeutic function of a protein is controlled not only by its primary chemical structure but also its secondary, tertiary, and in some cases quaternary spatial structures. Any subtle change in the three-dimensional structure may render the protein therapeutics ineffective. The structure of proteins has been discussed in detail in many biochemistry textbooks.¹ Here, we briefly review the structural characteristics of proteins.

The primary structure of a protein consists of a polypeptide chain with a specific sequence of amino acids connected by amide or peptide bonds. The polypeptide forms secondary structures including α -helices and β -sheets. The folding of the secondary structures with spatial dispositions of its side chains forms the tertiary structure of a protein. The tertiary structure is held together mainly by noncovalent interactions between side chains and sometimes by a covalent disulfide bond of cystine. In some cases, polypeptide

subunits associate in a specific way through noncovalent interactions among the subunits forming quaternary structure of a protein. The noncovalent interactions include: electrostatic interactions, hydrogen bonding, and hydrophobic interactions. The electrostatic interactions arise from the charge interactions (both attractive and repulsive) of the ionic side chains and van der Waals forces including dipole–dipole interaction of electrical neutral residues. Hydrogen bonding is the main force for the secondary structures, but internal hydrogen bonding seems having little effect on the stability of a tertiary structure. Hydrogen bonding with water molecules in solution stabilizes the tertiary structure. Hydrophobic interactions play a significant role in protein folding. The hydrophobic side chains of a polypeptide gather together to minimize their contact with water molecules. Hydrophobic interactions are considered as the driving force causing proteins to fold into their native three-dimensional structures.

The primary structure of a protein can only be changed by chemical reactions. Some functional groups of amino acid residues in a protein can be subject to chemical reactions, resulting in chemical degradation. The three-dimensional structure of a protein is relatively unstable and subject to destruction by many environmental factors via physical interactions, resulting in physical degradation or denaturation.

11.2.1 CHEMICAL DEGRADATION OF PROTEINS

Proteins are biochemicals with high molecular weights and their polypeptides are susceptible to various chemical reactions.^{2–4} The chemical reactions can take place via the interaction of a protein molecule with its environment including solvent (mainly water), other solutes, air, light, etc. These interactions may result in chemical degradation of a protein, the change of its primary structure. Figure 11.1 illustrates the possible chemical degradation pathways of proteins with representative amino acid residues.



FIGURE 11.1 Possible chemical degradation pathways of a polypeptide chain in a protein.

11.2.1.1 Hydrolysis

Hydrolysis is one of the main chemical degradation pathways of proteins. Amide bonds in polypeptide chains are considerably stable to hydrolysis under normal physiological conditions. However, they can be broken down at extreme pH by specific acid or base catalyzed hydrolysis. Deamidation and isomerization of asparaginyl residues are the major hydrolytic degradation reactions in proteins. The hydrolysis changes the asparaginyl residue into an aspartyl or isoaspartyl residue. The mechanism of deamidation and isomerization is shown in Figure 11.2. The hydrolysis of asparaginyl residue at low pH (pH < 2) mainly gives aspartyl residue by acid catalyzed hydrolysis of the amide in the side chain. At relatively high pH (pH = 5–12), the hydrolysis produces a mixture of isoaspartyl and aspartyl residues with a ratio about 3–4 to 1. The reaction can be catalyzed by both general base and specific base. A five-membered cyclic imide is formed as an intermediate, which is further hydrolyzed to give either aspartyl residue or isoaspartyl residue.

The rate of asparaginyl hydrolysis increases with increasing temperature. It is also affected by the local sequence around the aspartyl residue. The aspartyl residue is more stable when an amino acid residue with a bulky side chain is attached to its carboxylic group due to steric effect of the amino acid. For example, the half-life of the deamidation is 1.1 day at 37° C and pH 7.5 when a glycine is attached to the aspartyl residue in a short peptide. However, if the glycine is changed to a leucine, the half-life increases to 70 days. The rate of deamidation is also determined by the three-dimensional structure of a protein. The constraint of the secondary and tertiary structures renders the deamidation slower for a protein than a peptide.^{5,6}

Glutaminyl residues can hydrolyze in a similar mechanism to the hydrolysis of asparaginyl residues. The reaction takes place via formation of a sixmembered cyclic imide intermediate. However, it is about ten times slower than the hydrolysis of asparaginyl residues because the formation of a sixmembered cyclic intermediate is energetically less favorable than that of the



FIGURE 11.2 Deamidation and isomerization of asparaginyl residues.

five-membered succinimide. Aspartyl residue can also form the succinimide intermediate same as the asparaginyl residue. The hydrolysis of the intermediate gives isoaspartyl residues.

11.2.1.2 Racemization of Amino Acids

All the amino acids except glycine in proteins are L-amino acids. The chiral α -carbons of the amino acid residues are susceptible to racemization under basic conditions through a mechanism of base catalyzed tautomerization of carbonyl group of the amide bonds (Figure 11.3). An enolate intermediate is formed and the reverse reaction of the enolate gives a mixture of D- and L-amino acid residues. It has been observed that racemization of aspartyl residues in proteins are exceptionally faster, approximately five orders of magnitude faster than the free amino acid. The racemization of other residues in proteins is only two to four times faster than the corresponding free amino acid. It is proposed that the racemization of aspartyl residues involves a mechanism of formation of a cyclic imide intermediate same as shown in Figure 11.2. The α -carbon in the succinimide is more reactive towards nucleophilic attack of a base.

11.2.1.3 Oxidation

Several electron rich functional groups in proteins are potential sites for oxidation.⁷ These functional groups include: sulfhydryl in cysteine, disulfide in cystine, imidazole in histidine, thiolether in methionine, phenol in tyrosine, and indole in tryptophan. There are a number of mechanisms that may result in oxidative modification of amino acids, among them oxidation with atmospheric oxygen is considered as the major degradation reaction in formulations. The oxidation can be catalyzed by transition metal contaminants (e.g., Fe^{2+}/Fe^{3+} and Cu^+/Cu^{2+}) in buffer salts.^{8,9}

The sulfhydryl group can be oxidized to disulfide. Both sulfhydryl and disulfide groups can be oxidized stepwise to products of higher oxidation state including sulfenic acid (RSOH), sulfinic acid (RSO₂H) and sulfonic acid (RSO₃H). The oxidation of methionine gives methionine sulfoxide under mild oxidative conditions and sulfone under strong conditions. The oxidation of tyrosine is similar to that of phenols and may result in crosslinking with other functional groups in proteins. The oxidation of histidine and tryptophan mainly gives hydroxylated histidine and tryptophan,



FIGURE 11.3 Base catalyze racemization.

respectively, with the addition of a hydroxy group on the aromatic groups of the amino acid.

The oxidation of proteins can be prevented with antioxidants such as ascorbic acid and phenolic compounds. However, it has been observed that some antioxidants may promote autoxidation with metal impurities. For example, the combination of ascorbic acid and cupric impurities produces highly reactive hydroxyl free radicals with atmospheric oxygen, resulting in rapid oxidation of peptides and proteins.

11.2.1.4 Photodegradation

Some proteins are photosensitive and therefore exposure to light, particularly to ultraviolet light, can lead to photodegradation of proteins.¹⁰ Some light sensitive functional groups such as indole in tryptophan can adsorb energy from light illumination and form electronically excited species with high reactivity. These excited species may start a chemical reaction or transfer its energy to other species to start a new reaction. The most common photodegradation of proteins is photo-induced autoxidation. Under aerobic conditions, an excited species may transfer its energy to molecular oxygen to form highly reactive singlet oxygen and to start oxidation. The excited species may also dissociate to form highly reactive free radicals, which can react with molecular oxygen to start free radical chain oxidation. The photodegradation results in destruction of side chains of proteins, and intramolecular or intermolecular crosslinking of proteins. The amino acids susceptible to photooxidation are His, Trp, Met, and Cys.

11.2.1.5 Chain Cleavage at Asparaginyl and Aspartyl Residues

Peptide chain can be cleaved at asparaginyl and aspartyl residues in proteins. The reaction involves a different mechanism of formation of succinimide intermediate by the attack of the nitrogen in side chain to the peptide bond (Figure 11.4). The result of the reaction is cleavage of polypeptide chains and formation of two peptide chains. The chain cleavage at aspartyl residue occurs by a similar mechanism with the oxygen of the carboxylic group attacking the peptide bond. The reaction can take place at physiological pH and can be catalyzed by a base.

11.2.1.6 Degradation at Cystinyl Residues

The disulfide bonds of cystinyl residues connect two portions of a single polypeptide chain or bridge two independent chains in many proteins. The breakdown or incorrect pairing of the disulfide bonds can result in change of protein native structure.¹¹ The disulfide bond can be readily cleaved by free thiols via disulfide–thiol exchange reaction (Figure 11.5). Such exchange reaction may occur intramolecularly between a cysteinyl residue and a



FIGURE 11.4 Chain cleavage reactions at asparaginyl and aspartyl residues.

Disulfide-thiol exchange reaction



FIGURE 11.5 Disulfide-thiol exchange reaction.

cystinyl residue, particularly when proteins are under denaturing conditions. However, the exchange reaction is reversible and proteins can resume the native disulfide bonding under proper conditions. The disulfide bond can also be cleaved by specific acid and cleavage products then initiate disulfide exchange reaction (Figure 11.6). The disulfide bond exchange reaction can be inhibited by the presence of thiol scavengers such as maleimide and cupric ions.

The disulfide bond can be destroyed at high temperature at neutral pH or under strong basic conditions by base catalyzed β -eliminationn (Figure 11.7). A dehydroalaninyl residue is formed as a result of the β -elimination and it can



FIGURE 11.6 Acid catalyzed disulfide exchange reaction.



FIGURE 11.7 β -Elimination and cross-link reactions at cystinyl residues.

continue reacting with the amino group of lysine residues and thiol group of cysteinyl residues to form crosslinks via Michael addition. These reactions lead to permanent damage of a protein's native structure and loss of biological activity. β -elimination reactions have been observed in a number of proteins including lysozyme and bovine pancreatinic ribonucleases A.

11.2.2 PHYSICAL DEGRADATION OF PROTEINS

Physical instability of proteins refers to changes in their order (secondary, tertiary, and quaternary) strucutres, which may result in their denaturation, adsorption to surfaces, aggregation and precipitation. The threedimensional structure of proteins are retained by relatively weak noncovalent interactions. These interactions can be disrupted by physical and environmental factors, causing protein unfolding or denaturation. Unfolded proteins expose the hydrophobic portions of the polypeptides to solvent. The interaction between hydrophobic side chains and water molecules in solution is energetically unfavorable. The polypeptides tend to form aggregates through hydrophobic interactions.¹² When the aggregates reach large enough sizes, they precipitate from solution. In most cases, precipitation due to denaturation is an irreversible process and the protein precipitates are not biologically active. Physical degradation may cause much more rapid loss of biological activities of proteins than chemical degradation if they are not handled carefully. The factors affecting the physical stability of proteins are discussed below.

11.2.2.1 Effect of Temperature

Most proteins are stable at low temperature and less stable at high temperature. Protein denaturation at increased temperature can be reversible through a sharp transition over a small temperature range. The reversibility of protein thermal denaturation depends on the experimental conditions. Normally, higher temperature and longer heating time may cause irreversible denaturation. Thermal stability of a protein can be described by melting temperature (T_m) , the temperature at which 50% of protein molecules are unfolded. A higher T_m indicates a more stable protein structure. The thermal stability is also pH dependent. For example, T_m of RNase is 41°C at pH 2.8, 60°C at pH 5.8 and 64°C at pH 6.7.¹

11.2.2.2 Effect of pH

Proteins have ionizable functional groups and the ionization of these functional groups is pH dependent. Electrostatic status of proteins has a significant impact on the stability of their native structure. Most proteins have maximum thermodynamic stability and minimum solubility at or near the isoelectric point (pI) where the net charge is zero. A slight shift of pH from pI renders proteins moderately charged (either positively or negatively) and more soluble. However, further shift from pI to extreme pHs will increase like charges in proteins, resulting in electrostatic repulsion and increased tendency of protein unfolding and denaturation.

11.2.2.3 Effect of Salts

The concentration and nature of salts can have large effects on the stability of a protein and its solubility. According to the Hofmeister series, ions are ranked according to their ability to increase hydrophobic interactions and thus affect protein stability.1 The Hofmeister series show that anions in the order of SO₄²⁻, H₂PO₄⁻, AcO⁻, Cl⁻, Br⁻, I⁻, CIO₄⁻, and SCN⁻ tend to destabilize protein native structure, and the same holds true for the cations in the order of NH^{4+} , Cs^+ , K^+ , Na^+ , Li^+ , Mg^{2+} , Ca^{2+} , and Ba^{2+} . Salts of hard anions (e.g., ClO^{4-} and SCN^-), hard cations (e.g., Li^+ , Ca^{2+} and Ba^{2+}) or both decrease the stability of proteins. These ions and other agents including guanidine ions and urea causing protein denaturation are called chaotropic agents. High concentration of guanidine ions or urea is normally used for intentional denaturation, which may be reversible. These ions or agents interact strongly with functional groups of proteins and disrupt native noncovalent interactions, resulting in protein unfolding. On the other end of the series, salts of these ions (e.g., KH₂PO₄ and (NH₄)₂SO₄) have a stabilizing effect on protein native structure. In fact, saturated ammonium sulfate solution is used for reversible precipitation of proteins in protein

purification. It is also imperative to pay attention to the type of counterions used. For example, guanidinium hydrochloride is a strong denaturant of proteins, whereas guanidinum sulfate at equivalent concentrations can stabilize proteins.

11.2.2.4 Effect of Organic Solvents, Solutes and Detergents

The effect of water soluble organic solvents on protein stability in aqueous solution is concentration dependent.^{13,14} Organic solvents at high concentrations destabilize proteins by interfering the hydration shell of proteins and the hydrophobic interactions. Organic solvents at low concentration may have a stabilizing effect on proteins. Water soluble organic solutes and detergents have a similar concentration dependent effect on protein stability as the organic solvents. A relatively high concentration of sodium dodecylsulfate (SDS) is used in protein characterization as it causes complete unfolding of proteins.

11.2.2.5 Effect of Mechanical Processing

Proteins can be denatured by improper physical processing. For example, shaking and stirring create foaming of protein aqueous solution and increase in air/water interface. The air/water interface is hydrophobic and protein molecules at the interface tend to expose their hydrophobic residues to air. The exposure of hydrophobic regions in proteins initiates protein aggregation and precipitation by hydrophobic–hydrophobic interactions. Shearing forces applied to protein molecules also expose the hydrophobic regions in proteins, resulting in aggregation and precipitation.

11.3 PROTEIN STABILIZATION

When the mechanisms and causes of degradation of a protein are known, the simplest approach to stabilize the protein is to avoid the factors causing them. Generally, three approaches, including excipients or additives, chemical modification, and site mutagenesis, are employed to produce stable protein formulations.^{15–18}

Salts, polyalcohols and sugars, surfactants, and chelating agents are commonly used as excipients in protein formulations. Some ions can form ionic binding with proteins and therefore increase their physical stability. Polyhydroxyl organic compounds, such as glycerol, mannitol, and sugars, at low concentration increase protein stability by enhancing preferential hydration of proteins. A small amount of a surfactant or detergent can prevent aggregation by inhibiting intermolecular hydrophobic interactions and assist renaturation. Chelating agents form complexes with the chaotropic metal ions and catalytic ions, such as Cu(I), Cu(II), Fe(II), and Fe(III) ions. Complexation with chaotropic ions may prevent their interaction with proteins. However, chelating ligands should be carefully selected because some ligands may increase the catalytic activity of metal ions.

In addition to physical mixing with excipients proteins are often conjugated to water-soluble polymers, such as poly[(2-hydroxylpropyl) methacrylamide] or PEG. PEGylation of proteins is discussed at length in a Chapter 5. The polymer chains prevent physical contact of protein molecules and stabilize proteins by reducing denaturation and enzymatic degradation. The chemical modification can also modify the biological activity of proteins.

Site-specific mutagenesis is designed to increase protein stability by replacing amino acids at specific sites in a protein. Mutagenesis can stabilize a protein by improving its interior interactions. For example, replacing glycine to alanine increases core hydrophobicity, resulting in increased core packing density and decreased conformational flexibility. The stability of α -helices can be increased by monitoring the helix dipole through mutagenesis. The introduction of disulfide bond may stabilize protein's native conformation and prevent protein unfolding. The chemical stability of proteins can be improved by replacing the chemically susceptible side chains. For example, asparaginyl residue can be replaced by threonine or serine to avoid deamidation.

11.4 STABILITY OF NUCLEIC ACIDS

The biological activities of nucleic acids depend on the information stored in the sequence of linear polynucleotides. The structures of nucleic acids are not as complicated as those of proteins, and their higher-order structures are not as condensed as proteins. Only four repeat base units are in ribonucleic acids (RNA) and deoxyribonucleic acid (DNA). The bases are attached to the 1'-position of riboses or deoxyriboses and the sugar units are connected by phosphodiesters via the successive 3'- and 5'-positions to form linear polymer chains. The delicate linear structures of nucleic acids are susceptible to chemical and physical degradation.^{19–23} Improper physical agitation can cause fragmentation of the fragile nucleic acid chains. The chemical interactions may result in changes in the primary structures and cleavage of nucleic acid chains. The chemical degradation of nucleic acids was broadly discussed in the third issue of 1998 Chemical Reviews. This section will discuss main degradation pathways of nucleic acids.

11.4.1 DEGRADATION OF RNA

The main degradation pathway for RNA is hydrolysis, catalyzed by both acid and base.^{20,24} The susceptibility of RNA to hydrolysis is attributed to the presence of the 2'-hydroxyl group in the ribose residues. The degradation of RNA under acidic condition is relatively complicated. The acid catalyzed reaction gives chain cleavage products or an isomerization product, 3',5'-dinucleoside monophosphate. Two possible mechanisms are involved

in the acid catalyzed reaction based on the reaction pH (Figure 11.8). The rates of both acid catalyzed chain cleavage reaction and isomerization decrease with increasing pH at pH < 4. The rate of isomerization becomes pH independent at pH > 4. The rate of chain cleavage reaches the minimum around pH 5. Further increase in pH results in base catalyzed hydrolysis and increase in reaction rate. Chain cleavage reaction becomes dominant at pH > 8. Above this point, base catalyzed hydrolysis of RNA mainly produces

Acid catalyzed isomerization of RNA



Acid catalyzed chain cleavage of RNA



FIGURE 11.8 Specific acid catalyzed hydrolysis of RNA.



FIGURE 11.9 Specific base catalyzed hydrolysis of RNA.

chain cleavage products. The possible mechanisms of base catalyzed RNA hydrolysis are proposed in Figure 11.9.

Two possible mechanisms are involved in the specific base catalyzed degradation. Anionic 2'-alkoxide is formed as an intermediate in the mechanism A after proton abstraction by hydroxide. The alkoxide anionic intermediate is highly nucleophilic. In mechanism **B**, hydroxide assists the attack of the 2'-hydroxyl group on the phosphorus in a concerted way. The kinetics of specific based catalyzed RNA hydrolysis reported by Li and Breaker suggests that mechanism A is the most likely approach.²⁵ They studied specific cleavage of RNA phosphodiester linkage using a ³²P label chimeric DNA/RNA oligonucleotide, *pCGCTCACTATAGGAAGAGATG (A: ribonucleotide) under basic conditions (pH > 8). The reaction was first order with respect to the concentration of RNA. The oligonucleotide was cleaved at the site of ribonucleotide and the observed rate constant for the chain cleavage reaction increased with increasing pH in the range of pH 8–13. The rate reached a maximum when reaction pH was higher than pK_a (ca. 13.1) of the 2'-hydroxyl group (Figure 11.10). This is because when pH is higher than the pK_a , further increase in pH results in complete ionization of 2'-hydrxoyl group and the concentration of 2'-alkoxide remains the same as that of RNA. The reaction rate remains constant because there is no further increase of 2'-alkoxide concentration at very high pH. When pH is lower than the pK_a , the concentration of the 2'-alkoxide is pH dependent. Both the concentration and reaction rate increase with pH.

According to the pH-rate profile in Figure 11.10 and mechanism A, the concentration of the 2'-alkoxide anions depends on the pK_a of the 2'-hydroxyl



FIGURE 11.10 The pH-rate profile of specific base catalyzed hydrolytic chain cleavage of a chimeric DNA/RNA oligonucleotide, *pCGCTCACTATAGGAAGAGATG (<u>A</u>: ribonucleotide), at the ribonucleotide (adapted from Ref. 24). The solid line is the curve fit with Equation (11.5).

group and the reaction pH and the reaction rate changes with the concentration of 2'-alkoxide anions. At a constant pH, the rate equation of specific base catalyzed hydrolysis can be expressed as:

$$v = k_{\rm obs}[\rm RNA] \tag{11.1}$$

$$= k_{\rm OH}[\rm RNA-O^{-}] \tag{11.2}$$

where k_{obs} is the observed rate constant at constant pH; k_{OH} is the rate constant of specific based catalyzed hydrolysis; [RNA] and [RNA–O⁻] are the concentrations of total RNA and 2'-alkoxide anions, respectively. [RNA–O⁻] at a given pH can be calculated from the equilibrium of the ionization and mass balance:

$$[\text{RNA}-\text{O}^{-}] = \frac{K_{\text{a}}}{[\text{H}^{+}] + K_{\text{a}}} [\text{RNA}]$$
(11.3)

Therefore:

$$v = \frac{k_{\rm OH}K_{\rm a}}{\left[{\rm H}^+\right] + K_{\rm a}} [{\rm RNA}]$$
(11.4)

From Equations (11.1) and (11.4), the first-order rate constant is

$$k_{\rm obs} = \frac{k_{\rm OH} K_{\rm a}}{\left[\mathrm{H}^+\right] + K_{\rm a}} \tag{11.5}$$

The observed rate constant given by Equation (11.5) is pH dependent, which fits well with the data in Figure 11.10. When pH is higher than the $pK_a,[H^+] \ll K_a, k_{obs} = k_{OH}$, and the rate constant is pH independent because almost all RNA molecules are ionized at the 2'-hydroxyl position becoming RNA-O⁻. When pH is lower than the $pK_a,[H^+] \gg K_a$, Equation 11.5 becomes:

$$k_{\rm obs} = k_{\rm OH} K_{\rm a} / [{\rm H}^+] = k_{\rm OH} (K_{\rm a} / K_{\rm w}) [{\rm OH}^-]$$

The observed rate constant increases linearly with increasing pH in the range of 8 to pK_a . The activation energy for specific base catalyzed RNA hydrolytic cleavage is about 29 kcal/mol. The reaction rate is also affected by ionic strength and catalytic metal ions. It is observed that the hydrolysis rate increases with increasing concentration of potassium ions. The presence of magnesium ions also accelerates the hydrolysis. Magnesium ion is a Lewis acid and acts as a co-catalyst.

General acids and general bases can catalyze the chain cleavage and isomerization of RNA.^{26,27} It has been reported that imidazole buffer leads to the hydrolytic cleavage of RNA. The reaction rate increases at high imidazole concentration and varies with the ratio of protonated imidazole at a fixed total imidazole concentration. In RNase A two essential histidine residues are involved in the enzymatic cleavage of RNA. The imidazole groups act as general acid and general base catalysts in a concerted way, accelerating the cleavage of RNA chains.

Other functional groups including bases and riboses in RNA molecules can be subjected to chemical degradation. However, the degradation rate of these groups is much slower than hydrolysis of phosphodiesters. The chemical degradation of these groups is normally considered insignificant in RNA degradation. The mechanisms of chemical degradation of bases and riboses in RNA are similar to those in DNA, which are discussed in the following section.

11.4.2 DEGRADATION OF DNA

DNA is stable to hydrolytic chain cleavage due to the absence of 2'-hydroxyl group in the deoxyriboses. Chemical degradation of bases and deoxyribose is the major concerns for chemical stability of DNA.²⁸ The chemical degradation pathways include the hydrolysis of bases, oxidation, and photochemical reactions. Physical instability of DNA is also a major concern for DNA pharmaceuticals.

11.4.2.1 Hydrolysis of DNA

Phosphodiesters in DNA molecules are stable towards hydrolysis. However, N-glycosyl bonds in DNA are susceptible to hydrolysis, resulting in loss of bases and further degradation reactions. Hydrolysis of N-glycosyl bonds can be catalyzed by acid and base. The rate of hydrolysis is structure dependent.^{29–31} Hydrolysis of N-glycosidic bonds with purines including adenine and guanine, depurination, is approximately 20 times faster than that of depyrimidination, hydrolysis of N-glycosidic bonds with pyrimidines. Under acidic condition, purine base is protonated and the N-glycosyl bond is weakened. The N-glycosyl bond is irreversibly broken by the neighboring oxygen atom, giving a free purine base and apuric DNA (Figure 11.11).

Two possible mechanisms are involved in base catalyzed depurination (Figure 11.12). Hydroxide attacks either C-8 of the purine (addition) or C-1' of the deoxyribose (S_N 2 substitution). The addition of hydroxide to the C = N double bond in imidazole ring initiates ring opening and cleavage of the N-glycosyl bond. In S_N 2 substitution, the purine is directly replaced by a hydroxyl group. The mechanisms of acid and base catalyzed depyrimidination are similar to those of depurination.

The rate of depurination is accelerated by both specific acid and specific base at high concentration. DNA has highest stability towards depurination at pH 8–9. Depurination is more rapid in the solution of lower ionic strength, possibly due to increased reactivity of specific acid and base. High temperature facilitates depurination and the activation energy of depurination is approximately 30 kcal/mol. Depurination of double-stranded DNA is approximately four times slower than that of single-stranded DNA.

Apurinic DNA is not stable and susceptible to further hydrolysis, resulting in the cleavage of DNA chains.³² For example, apurinic DNA has a semi-acetal residue, which reversibly forms aldehyde. The protons on C-2'



FIGURE 11.11 Acid catalyzed depurination.



FIGURE 11.12 Base catalyzed depurination.

are relatively acidic and a base can attack these protons resulting in chain cleavage via β -elimination (Figure 11.13). It has been observed that the rate of β -elimination is much faster than that of depurination. It can quickly lead to chain cleavage after depurination.

Another hydrolytic degradation pathway of DNA is deamination, hydrolysis of the amino group on purines and pyrimidines.³³ Deamination is insignificant in the chemical degradation of purines because the rate of deamination of purines is about three orders of magnitude slower than that of depurination. Cytosine is more susceptible to deamination than other base residues. Deamination of cytosine shows small pH dependence at pH 6–9 and strong pH dependence above or below this pH range. Both acid and base can catalyze the deamination and possible mechanisms of catalyzed deamination are described in Figure 11.14. Under acidic condition, the N-3 is protonated, facilitating the nucleophilic attack of water the C-4 followed by elimination of the amino group. Under basic condition, hydroxide directly attacks the C-4 followed by deamination. The deamination converts cytosine to uracil. Activation energy of cytosine deamination at pH 7.4 is 29 kcal/mol. High temperature increases the rate of demination. Deamination in double stranded DNA is about 150 times slower than that of single-stranded DNA.



FIGURE 11.13 Hydrolytic chain cleavage at the apurinic site of DNA.



FIGURE 11.14 Acid and base catalyzed deamination of cytosine.

11.4.2.2 Photodegradation of DNA

Two major mechanisms are involved in photodegradation of DNA. One involves the photo-induced generation of free radicals followed by free radical oxidation under aerobic condition. This is discussed along with oxidative degradation in the next section. The presence of photosensitizers promotes photo-induced autoxidation. The other mechanism involves photochemical crosslinking. Double bonds in the base residues can be excited by the light irradiation and lead to crosslinking of bases via 2+2 cycloaddition (Figure 11.15). The reaction can be reversed thermochemically.

11.4.2.3 Oxidation of DNA

Oxidation is another major degradation pathway for the chemical degradation of DNA.^{34,35} Oxidation with atmospheric oxygen, autoxidation, is a common



FIGURE 11.15 Photolytic crosslinking of pyrimidines.

oxidation pathway of DNA pharmaceuticals. Autoxidation is a free radical process, which can be initiated by free radical initiators, photolysis, or transition metal ion catalyzed redox reactions. It has been observed that impurities of transition metal ions in buffer salts can result in significant chain cleavage of plasmid DNA. The chain cleavage can be slowed down by proper chelating agents and free radical scavengers. Fe³⁺, Fe²⁺, Cu²⁺, and Cu⁺ are the common transition metal impurities in buffer salts. Hydrogen peroxide and alkyl hydroperoxide can undergo redox reactions with these metal ions to generate reactive hydroxyl or alkoxyl free radicals to initiate free radical chain auotoxidation. Hydroperoxides may derive from aged excipients or direct autoxidation of electron-rich organic functional groups, such as the base residues in DNA. The redox reaction ion pairs Fe^{3+}/Fe^{2+} and Cu^{2+}/Cu^{+} in the presence of molecular oxygen can also produce oxidative free radicals. The presence of reducing agents may facilitate the metal catalyzed production of reactive free radicals from atmospheric oxygen. Figure 11.16 shows the possible reactions generating reactive hydroxyl radical.

Once a reactive free radical species is generated, it can attack both nucleobases and deoxyriboses. Figure 11.17 shows the hydroxyl free

$$H_{2}O \xrightarrow{hv} HO\bullet + H\bullet$$

$$2 \operatorname{Fe}(II) + O_{2} + 2H^{+} \longrightarrow 2 \operatorname{Fe}(III) + H_{2}O_{2}$$

$$\operatorname{Fe}(II) + H_{2}O_{2} \longrightarrow \operatorname{Fe}(III) + HO^{-} + HO\bullet$$

$$\operatorname{Fe}(II) + ROOH \longrightarrow \operatorname{Fe}(III) + RO^{-} + HO\bullet$$

$$2 \operatorname{Cu}(I) + O_{2} + 2H^{+} \longrightarrow 2 \operatorname{Cu}(II) + H_{2}O_{2}$$

$$\operatorname{Cu}(I) + H_{2}O_{2} \longrightarrow \operatorname{Cu}(II) + HO^{-} + HO\bullet$$

$$\operatorname{Cu}(I) + ROOH \longrightarrow \operatorname{Cu}(II) + RO^{-} + HO\bullet$$

FIGURE 11.16 Generation of hydroxyl free radicals.



FIGURE 11.17 Oxidation of guanine.

radical initiated oxidation of guanine, which gives 8-oxo guanine. Adenine is similarly oxidized to give 8-oxo adenine. There are three possible pathway of hydroxyl free radical induced oxidation for thymine (Figure 11.18). Cytosine and uracil are similarly oxidized via pathways 1 and 2. The oxidized nucleobase is a better leaving group for hydrolysis, which leads to depurination or depyrimidination and subsequent chain cleavage.

The autoxidation of deoxyriboses depends on the reactivity and accessibility of hydrogen atoms on the deoxyriboses to hydroxyl free radicals. Figure 11.19 summarizes the reactivity of various hydrogen atoms in the deoxyriboses towards free radical abstraction and their contribution in oxidative degradation of duplex DNA. Hydrogen atoms at the 4'- and 5'-positions have the most accessibility to solvents and reactants and are most likely to be attacked by free radicals non-specifically. The autoxidation at these positions contributes more oxidative degradation than that at the



FIGURE 11.18 Oxidation of thymine.



FIGURE 11.19 The reactivity of abstract of hydrogen at the deoxyribose residue and contribution in oxidative DNA degradation.

other positions. Figure 11.20 and Figure 11.21 illustrate the possible autoxidation of DNA after the abstraction of the protons at the 4'- and 5'-positions, respectively. Both autoxidation pathways result in DNA chain cleavage.



FIGURE 11.20 Oxidative chain cleavage initiated by abstraction of 4'-hydrogen.



FIGURE 11.21 Oxidative chain cleavage initiated by abstraction of 5'-hydrogen.

11.4.3 PHYSICAL STABILITY OF NUCLEIC ACIDS

DNA mostly has secondary structure and only plasmid DNA has tertiary structures, the supercoiled structure. Plasmid DNA is commonly used as gene therapeutics. If one of the double strands breaks, supercoiled plasmid DNA becomes an open circle.³⁶ If both strands are broken at close vicinity, then plasmid DNA forms linear DNA. The biological activity of plasmid varies with its structure. The open circular plasmid preserves approximately 90% of transfection efficiency of the supercoiled plasmid, while the linear plasmid only has about 10% of its original efficiency. The strand breakage is usually the result of chemical degradation or physical agitation. Mechanical forces during formulation process can also cause DNA chain cleavage.³⁷ Vigorous stirring or shaking of solution of naked DNA can break down the delicate DNA chain structure. It has been observed that ultrasonication of naked plasmid DNA results in significant fragmentation in 15 sec and complete fragmentation in 30 sec. Condensation with polycationic materials increases the physical stability of plasmid DNA.

Chemical degradation of DNA can be significantly slowed down at low temperature and in frozen state. However, improper freezing-thawing process may cause significant DNA degradation. It is believed that formation of cracks in the ice during freezing causes strand to break. Fast cooling rates generate more ice cracking and consequently more chain breaks of naked DNA. In the case of condensates of plasmid DNA with cationic substances, it is observed that transfection efficiency is maintained during rapid cooling and reduced during slow cooling. The freezing-thawing process also results in aggregation of condensates and consequently low transfection efficiency. Excipients, like sugars, are frequently used to reduce strand cleavage during freezing.

11.5 STABILIZATION OF NUCLEIC ACIDS

The degradation of RNA mainly involves the 2'-hydroxyl group assisted chain cleavage and isomerization. The substitution of the 2'-hydroxyl group with a less nucleophilic functional group remarkably increases RNA stability to hydrolysis. Polynucleotides of 2'-amino-2'-deoxyuridine can stand treatment with 0.1 M KOH aqueous solution at 37° C for 80 min, whereas 90% of the unmodified poly(U) is cleaved. This is due to the low nucleophilicity of the animo group in comparison with alkoxide under strong basic conditions. Similarly, the replacement of the 2'-hydroxyl group with less nucleophilic sulfhydryl group results in stable RNA. It has been reported that neither cleavage nor isomerization is observed for 2'-deoxy-2'-thiouridylyl(3',5')uridine in 0.1 M HCl at 100°C for 1 hr.

The major degradation pathways for DNA are depurination followed by β -elimination and autoxidation. Depurination is pH dependent and DNA has the maximum stability between pH 8–9. DNA molecules have reasonable stability in aqueous solution at pH 8.5 and room temperature if oxidative degradation is properly controlled. The storage of DNA solution at low temperature (above freezing temperature) can significantly decrease the rate of chemical degradation. It has been reported that supercoiled plasmid DNA is stable for two years at pH 7.4 and 5°C, while storage at pH 7.4 and 30°C resulted in significant degradation in less than half year.

Oxidative degradation is a free radical process and catalyzed by transition metal ions. The addition of free radical scavengers including ethanol and phenols can turn highly reactive hydroxyl free radical into less reactive free radical and terminate free radical chain autoxidation. It is ideal to use buffer solution free of contamination with transition metal ions. Metal contamination can be effectively removed by using chelating resin. Chelating ligands may also be used if the solution is contaminated. However, chelating ligand alone cannot effectively prevent autoxidation and improper selection of ligands may promote autoxidation.

11.6 CONCLUDING REMARKS

Instability of proteins and nucleic acids is a complicated issue. There are always interactions of these pharmaceuticals with their surrounding environment during the formulation process and in their final formulations. The interactions may cause significant deterioration of the formulations over time period. It is impossible to avoid such interactions and to completely prevent the degradation of the formulated proteins and nucleic acids. However, if we understand the major degradation pathways for proteins and nucleic acid, the factors causing their degradation can be avoided in the formulation
process and in their final formulations. The degradation can be delayed to prolong their shelf-lives, which will allow transport and storage without significant loss of biological activity.

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12 Formulation, Stability, and Characterization of Protein and Peptide Drugs

Sven Frokjaer, Lars Hovgaard, and Marco van de Weert

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12.1 INTRODUCTION

The evolution of recombinant DNA technology and the advances in proteomics render the production and use of biologically-based therapeutics as one of the most challenging and rapidly growing areas within pharmaceutical sciences. The primary objective of pharmaceutical formulation is to formulate drug compounds into products with optimal therapeutic effect and shelf-life. Although this objective holds for all drugs, the formulation of protein and peptide drugs is more complex and demanding. The therapeutic application of proteins is limited by several problems, such as potential physical and chemical instability as well as limited transport across biomembranes (Figure 12.1). Thus, the pharmaceutical scientist is confronted with the challenge of formulating therapeutic proteins and peptides into safe, stable, well-characterized and efficacious drug products. In this chapter the formulation, stability, and analytical aspects of pharmaceutical proteins and peptides will be discussed. For a more comprehensive overview on pharmaceutical formulation aspects of biotechnological products, references are made to a few recent textbooks.^{1,2} A recent comprehensive list of protein and peptide drugs on the market has been provided by Walsh (2003).³ The list includes:

- Recombinant blood factors/blood related products
- Recombinant hormones
- Cytokines
- Vaccines
- Monoclonal antibody-based products
- Therapeutic enzymes and additional products



FIGURE 12.1 Transport of drugs across a mucous cell membrane.

12.2 PHARMACEUTICAL FORMULATION

The successful formulation design of proteins depends on the understanding of their physicochemical and biological characteristics, which include molecular weight, chemical and conformational stability, immunogenicity, biological half-life, and pharmacokinetic properties. Although the oral route of administration is the preferred and most widely used route of drug administration, mainly due to patient compliance, this route is generally not feasible for the delivery of proteins and peptides. First, the inherent instability of proteins and peptides, which is due to chemical degradation and metabolism, may occur at several sites in the gastrointestinal tract, e.g., in the lumen and at the brush border as well as intracellularly. Second, the permeability of high molecular weight and polar molecules through biological membranes is very low. Thus, most protein and peptide drugs are administered parenterally, although efforts are made to enhance systemic bioavailability by means of alternative routes of administration, e.g., nasal, pulmonary, and transdermal routes.

12.2.1 FORMULATIONS FOR PARENTERAL ADMINISTRATION

Almost all therapeutic protein and peptide drugs for parenteral use are formulated either as suspensions or aqueous solutions in a ready-to-use form, or as a freeze-dried powder for reconstitution. Depending on the actual disease and the pharmacokinetic properties of the protein the product can be administered by various parenteral routes, e.g., intravenous, intramuscular, or subcutaneous.

There are some fundamental guidelines to consider in the development of any protein formulation. These are summarized below:

1. The formulation has to be sterile and if possible iso-osmotic to biological fluids, and have a pH around 7.4. If formulated as a solution it should be particle-free. This means that the effects of temperature, pH, buffer type and concentration, and ionic strength must be known. This information as well as an understanding of other physical and chemical properties, e.g., effect of oxygen and mechanical stress, are obtained from preformulation studies. In general, proteins are sensitive to heat and cannot withstand terminal sterilization. Therefore, protein-based products as well as most peptides have to be produced under aseptic conditions, normally including an aseptic filtration step. The effect of flow rate during filtration and filling must be understood and a strict control of bioburden must be maintained to ensure that the final product is essentially endotoxin-free. Since recombinant DNA products are produced in micro-organisms and often by using biological material as part of the substrate, it is important to ensure that microbial or viral contamination does not occur.

- 2. Apart from the active protein or peptide, a number of excipients can be used in the formulation. They can serve various purposes, e.g., as solubility enhancers, anti-adsorption agents, buffer components, osmotic agents, preservatives, antioxidants, and, in freeze dried products, as lyoprotectants.
- 3. The container and closure systems should be compatible with the formulation. Proteins are well known to be interfacially active. Therefore, they tend to adsorb onto surfaces including glass-water and plastic-water, and concentrate at the air-water interface, reducing the total amount of therapeutically available protein in the solution. This interfacial activity may also initiate denaturation or fibrillation leading to a loss in biological activity. Plastic containers and closure systems may leach some of their additives into the drug product or absorb excipients from the formulation, potentially reducing the shelf-life of the formulation.
- 4. Stability studies must be conducted to establish the shelf-life of the final product. During those studies the effects of temperature, mechanical stress, and exposure to light are critical parameters, which need to be investigated.

12.2.2 NON-PARENTERAL ROUTES OF ADMINISTRATION

Delivery of protein and peptide drugs by the nonparenteral route is useful for specific local treatments. However, there are many obstacles to nonparenteral administration of proteins and peptides for systemic use. These obstacles may broadly be categorized as (a) enzymatic barriers that exist at the site of administration or as part of the transport pathway to the site of action, and (b) physical barriers to the transport of drug substances through the epithelium. Numerous reviews on potential nonparenteral routes of administration of protein drugs can be found in literature.⁴

The different anatomical and physical characteristics of the various barriers, e.g., nasal, buccal, transdermal, pulmonary, ocular, vaginal, and rectal barriers, set the limits for absorption. Therefore, the choice of administration site for a specific disease relies on a complete evaluation of drug characteristics, membrane properties, and the type of treatment. The nasal mucosa, for instance, is highly vascularized, and the absorptive epithelium consists of only one cellular barrier. Therefore, the possibility of a rapid systemic delivery of proteins and peptides exists despite a limited and potentially variable residence time in nasal cavity.^{5,6} Compared to the nasal route, the buccal route is less effective with respect to transport due to a thicker ceratinized multilayer epithelium, although the lack of tight-junctions between the epithelial cells still opens for systemic absorption of hydrophilic macromolecules.⁷ Potential advantages of the transdermal protein and peptide delivery are the ease of administration, the possibility of long-term application, and excellent patient compliance. Although the skin is extremely

TABLE 12.1 Important characteristics for non-parenteral routes of delivery for peptide drugs*

Route of delivery	Absorption area	Predicted absorption	Proteolysis	Accessibility	Hepatic first pass	
Oral	$200\mathrm{m}^2$	0.1%	+++++++++++++++++++++++++++++++++++++++	+++	High	
Rectal	$200-400 \mathrm{cm}^2$	0.1%	+++	++	Partly avoided	
Buccal	$100\mathrm{cm}^2$	1%	++	++	Avoided	
Nasal	$150\mathrm{cm}^2$	5%	++	++	Avoided	
Pulmonal	$70 \mathrm{m}^2$	50-100%	0	+	Avoided	
Transdermal	$2 \mathrm{m}^2$	1%	++	+++	Avoided	
[*] Modified from Crommelin and Sindelar ² ; Ingemann et al. ⁴						

impermeable, the transdermal route offers future possibilities for systemic delivery of peptides, e.g., by use of iontophoresis or other electrostimulusinduced absorption processes.^{8,9} More recently, the pulmonary route has received significant attention as it is relatively easy to access and shows a fast systemic uptake due to the large area available for absorption combined with a thin epithelial membrane.¹⁰ However, a major pharmaceutical challenge in pulmonary delivery of protein and peptide drugs lies in an effective deposition of aerosol particles in the alveoli.

In general, the bioavailability via the various nonparenteral routes of administration is too low for an effective systemic effect. The pulmonary route may be the exception as insulin formulations for pulmonary administration are now in Phase 3 clinical trials. The potential advantages and disadvantages of alternative routes of administration to the oral route are listed in Table 12.1.

12.3 DELIVERY OF PROTEINS AND PEPTIDES

12.3.1 CHEMICAL MODIFICATION OF PROTEINS AND PEPTIDES

The properties of proteins may be optimized with respect to bioavailability and pharmacokinetic properties as well as chemical and physical characteristics which are of importance to the pharmaceutical formulation. Two approaches may be used; either a permanent chemical modification of the molecule, i.e., a protein or peptide analogue, or a bioreversible derivatization of the protein or peptide, i.e., a prodrug.

For peptides, stability against enzymatic degradation may be optimized by chemical modification, e.g., by substituting one or more L-amino acids with D-amino acids, or by methylation of the α -amino group in the peptide bond.¹¹ The design of peptidomimetics is another attractive approach for optimization of the drug delivery potential of smaller peptides.¹² This approach or a prodrug approach may also be used to design compounds as substrates for the human intestinal peptide transporter, hPepT1, in order to improve oral bioavailability.^{13,14} For proteins, the analogue approach is by far the most frequently applied strategy. Insulin provides an excellent example to this approach and is discussed below.

Rapid-Acting Insulin Analogues 12.3.1.1

The pharmacodynamics of soluble human insulin formulations show an onset of action approximately 0.5 hr after subcutaneous administration. The peak value is reached around 2 to 3 hr and the time of duration is 6 to 8 hr. As with other formulations, the pharmacodynamics may vary depending on a number of factors, such as dose, injection site, and the patient's physical activity. Despite the fact that insulin is already dissolved, a delay in action is still observed due to the time required for the insulin hexamer to dissociate through a dimeric insulin form to the biologically active monomeric insulin molecule, followed by transport from the injection site across the biological membrane into the systemic circulation. A schematic illustration of the absorption process of human insulin is given in Figure 12.2.

The development of rapid-acting monomeric analogues of insulin for treatment of type-1 diabetes was first suggested by Brange et al. (1990).¹⁵ The hypothesis behind this development was that by shifting the self-association characteristics of insulin in favor of monomeric rather than hexameric insulin, the delay in onset of action could be reduced.¹⁶ Such monomeric analogues with a more rapid time-action profile and peak activity after approximately 1 hr are now on the market (insulin lispro (Eli Lilly and Company) and insulin aspart (Novo Nordisk A/S)). Recently, Aventis has submitted a marketing authorization application for the rapid-acting insulin analogue *Glulisine* in the European Union and the United States.



Insulin dissociation state

FIGURE 12.2 Schematic representation of insulin membrane transport in relation to association state.

12.3.1.2 Acylated Peptides

The use of acylated peptides has received increasing attention over the last decade.^{17–20} By increasing the lipophilicity of peptide drugs, it may be possible to overcome some of the inherent problems associated with peptide drug delivery. As an example, bioreversibly acylated desmopressin administered subcutaneously to rats shows a 250-fold increase in the anti-diuretic potency as compared to unmodified desmopressin. This increase in potency is most likely due to a decreased elimination rate and prolonged tissue retention.¹⁹ Additionally, acylation of insulin dramatically prolongs the circulation time after subcutaneous administration due to binding to the circulating serum albumin.^{18,21}

A long-acting insulin analogue, [Lys.sup.B29]-tetradecanoyl des(B30) human insulin, insulin detemir (Novo Nordisk A/S), has very recently been approved by authorities in some countries. Using the principle of acylation to prolong the action of drugs by facilitating binding to serum albumin has also been used for a number of other peptides, e.g., glucagon-like peptide-1²⁰ and interferon- α .²²

12.3.1.3 PEGylation of Proteins

The elimination half-life of many proteins and peptides is rather short. Consequently, it may be necessary to administer the drug by continuous infusion or by formulation of suitable controlled release systems. A number of different strategies have been applied to reduce the clearance rate. One approach, which has gained significant interest, is chemical modification of proteins and peptides with polyethylene glycol (PEG), i.e., PEGylation. There is an in-depth discussion on PEGylation of proteins in Chapter 5. Besides reducing clearance rate by preventing recognition and degradation by proteolytic enzymes and shielding receptor-mediated uptake by for instance the reticuloendothelial system (RES), the reasons for PEGylation of peptides and proteins include an improved safety profile and tolerability by the shielding of antigenic and immunogenic epitopes.

An example is the 12 kD linear PEGinterferon α -2b which is approved for treatment of chronic hepatitis C. The plasma clearance rate of unmodified interferon α -2b is approximately 6 to 7 hr, while the half-live for PEGinterferon α -2 is approximately 40 hr. The product consists of a mixture of interferon molecules with one or more PEG polymers attached via the ε -amino group of lysine residues. Due to safety and efficacy reasons it is important that drugs are well-defined, e.g., the number of PEG-polymers attached should be known. However, it is also important to pay attention to the fact that the PEG-polymer consists of a mixture of polymers with different molecular weight and that any coupling process results in a mixture. Due to this product heterogeneity, the chemical and pharmaceutical documentation as well as the safety and clinical documentation may be even more demanding than for



FIGURE 12.3 Illustration of the prodrug principle.

nonmodified drugs. Excellent reviews are available on these specific types of derivatives.²³⁻²⁵

12.3.1.4 Prodrugs of Peptides

A prodrug is defined as a pharmacologically inactive derivative of a drug molecule that is degraded quantitatively into the pharmacologically active parent molecule, either spontaneously or enzymatically, in the body (Figure 12.3). The progroup or promoiety should be nontoxic.

During the past two decades various approaches have been made to improve oral bioavailability and reduce enzymatic degradation of peptides in the gastrointestinal tract and other biological compartments, e.g., in blood.

A potential strategy for modifying the α -amino-amide moiety, which is present in most peptides, is to form 4-imidazolidinones, by condensing the α -amino-amide moiety in the peptide bond with a free N-terminal amino group, to aldehydes or ketones.²⁶ N-alkoxycarbonyl prodrugs of the imidazole group of the histidine residue are another example in which in vitro tests have shown improved stability toward enzymatic degradation.²⁷ More recently, Borchardt and co-workers have developed the prodrug approach further by a number of studies of cyclic peptide-prodrugs using various linkers for cyclization, e.g., using acyloxyalkoxy or phenylpropionic acid as promoities.²⁸

12.3.2 PROTEIN AND PEPTIDE DELIVERY SYSTEMS

Entrapment and encapsulation are the most widely used pharmaceutical techniques when designing drug delivery systems for proteins. Examples are polymeric drug delivery systems, such as hydrogels, nanocapsules, and microspheres, and lipid-based drug delivery systems, such as liposomes and solid lipid nanoparticles.

Hydrogels are by definition cross-linked hydrophilic polymers which are able to swell in aqueous environments. The degree of swelling can be controlled by the type of polymer, the degree of cross-linking and the character of the aqueous medium, e.g., pH, ionic strength, and temperature. They exhibit a range of chemical, physical, and biological characteristics including biocompatibility which make them attractive as drug delivery systems. The release characteristics of entrapped proteins or peptides are either controlled by diffusion through the hydrogel matrix or by degradation of the polymer.

As for hydrogels, microencapsulation both in microcapsules and microspheres can protect proteins and peptides from enzymatic degradation, thereby offering a potential controlled drug release from the site of administration over a prolonged period of time. In literature, there are numerous examples of polymers for controlled release purposes, including both synthetic and natural polymers. Presently, there are microencapsulated peptides (e.g., triptorelin, which is a synthetic gonadotropin-releasing hormone analogue) and proteins (e.g., human growth hormone) on the market, formulated within/using PLGA (polylactide-glycolic acid) and with a release period of 4 weeks/30 days.

Examples of lipid-based formulations with the potential of serving as delivery systems for proteins and peptides include liposomes,²⁹ solid lipid nanoparticles³⁰ and water-in-oil emulsions.³¹

12.4 STABILITY OF PROTEINS AND PEPTIDES

Maintenance of the chemical and structural integrity of a protein or peptide drug is absolutely essential for its efficacy in relation to its pharmacological activity and safety profile. Therefore, the stability aspect of drugs is of major importance to all formulation scientists. Unlike small organic molecules, proteins and peptides possess not only a primary structure determined by the amino acid sequence, but also higher-order structures. Thus, stability problems related to small peptides involve primarily chemical degradation. For proteins and larger peptides, the degradation pathways include chemical degradation similar to that observed in peptides, as well as structural (physical) instability. In contrast to chemical degradation, physical instability does not involve covalent modification of the protein or peptide. Readers are encouraged to refer to Chapter 11 for a detailed discussion on protein stability.

12.4.1 CHEMICAL DEGRADATION OF PROTEINS AND PEPTIDES

The major pathways of peptide and protein degradation are hydrolysis, oxidation, racemization/isomerization, β -elimination, and disulfide exchange (Table 12.2). Among the 20 naturally-occurring amino acids, Asn, Gln, Met, Cys, His, Trp, and Tyr are the most labile.

The most common deamidation reaction in proteins and peptides involves the side chain amide bond in Asn and Gln. The complexity of the

TABLE 12.2 Degradation of various amino acids [*]					
Degradation pathway	Amino acid examples				
Hydrolysis	Asn and Gln (side chains)				
	Asp and Pro (from N-terminal)				
	Met and Cys (at the sulfur site)				
Oxidation	His, Tyr and Trp (in the aromatic rings)				
	Pro				
Racemization/isomerization	Asp				
β-Elimination	Cys, Ser, Thr, Phe and Lys				
Disulfide exchange	Cys				
*From Goolcharran et al. (2000). ⁴⁷					

deamidation reaction can be illustrated by the pathways for the deamidation of Asn (Figure 12.4). The relative importance of the different pathways is dependent on experimental conditions, such as pH³² as well as the amino acid sequence³³ and the tertiary structure of the molecule.³⁴

Deamidation of Asn residues at neutral and alkaline pHs is generally accepted to go via a cyclic imide intermediate, formed by the intramolecular attack of the α -amino group, to the succeeding residue on the side-chain carbonyl group of the Asn residue. This intramolecular imide is subsequently hydrolyzed into a mixture of different desamindo products in which the peptide backbone is linked through an α -carboxyl linkage (Asp-derivative) or a β -carboxyl linkage (isoAsp-derivative). The cyclic imide can undergo racemization at the α -carbon, generating the corresponding D-Asp and D-isoAsp residues (Figure 12.4).

Several mechanisms are involved in the oxidative degradation of peptides and proteins.³⁵ The underlying reactions in peptide and protein oxidation involve the formation or presence of oxidative species. The various oxidation mechanisms can be categorized as autooxidation, metal-catalyzed oxidation and photooxidation. Factors such as pH, temperature, trace amount of metal ions (e.g., Fe and Cu), and buffer can affect the rates of oxidative reactions. A typical oxidative reaction can be represented by the oxidation of Met (Figure 12.5) to methionine sulfoxide derivatives and, under strong oxidizing conditions, even further to sulfone derivatives.

The localization of oxidation-labile amino acids in the protein or peptide structure, i.e., whether it is localized on the exterior or buried in the structure, may have a significant effect on the oxidation potential, especially for oxidation processes initiated by external reactive oxygen species. However, for metal-catalyzed oxidation this is not a significant factor, because the oxidation will only occur around the metal-binding sites.³⁶

In pharmaceutical formulations the external reactive oxygen species may arise from contaminants in excipients used in the formulation, e.g., peroxides in surfactants.³⁷ Even the addition of an antioxidant, such as



FIGURE 12.4 Deamidation of Asn residues. (Adapted from Goolcharan et al., 2000).⁴⁷



FIGURE 12.5 Oxidation of methionine.

ascorbic acid, may accelerate the oxidation process in the presence of trace amounts of transition metals. 38,39

Several approaches can be taken to optimize the chemical stability of a protein or peptide drug and thereby improve shelf-life. In general, solid-state

formulations, such as suspensions or lyophilized products, are more stable than liquid formulations. For liquid formulations, the composition of the aqueous medium should be optimized according to pH, buffer, osmotic agents, co-solvents, surfactants, antioxidants, preservatives, etc. However, at the same time the formulation should fulfill the demands both from a physiological, therapeutic, and a regulatory point of view. In practice, this often means that the final formulation is an "optimized" compromise. The influence pH has on the formation of various degradation products during storage of insulin crystals, e.g., mono- and didesamido insulin, insulin split product and di- and oligomers of insulin, may serve as an example of the complexity in optimizing a pharmaceutical formulation from a chemical stability point of view (Figure 12.6).⁴⁰

12.4.2 PHYSICAL STABILITY OF PROTEINS AND LARGER PEPTIDES

In contrast to low molecular weight compounds, proteins and larger peptides are, in addition to chemical degradation, also susceptible to changes in the three-dimensional structure, i.e., (partial) unfolding, denaturation, aggregation, fibrillation, adsorption onto surfaces, and precipitation. In contrast to chemical instability, physical instability does not involve covalent bond-cleavage of the molecule. As for chemical stability, it is essential to understand the physical stability issues relevant for production, formulation, storage and use of protein and peptide drugs.

Denaturation can either be reversible or irreversible, and it refers to an alteration of the global structure of the native molecule resulting in a reduced or altered activity. Factors potentially affecting denaturation of proteins and peptides are temperature, pH, ionic strength, various excipients (e.g., surfactants and organic solutes), organic solvents, and mechanical stress. Proteins and peptides may self-associate, resulting in formation of non-native dimers, oligomers and macromolecular aggregates that remain in the solution. As for denaturation, self-association or aggregation depend on factors such as temperature, pH, and solvent composition. Aggregation kinetics are often described by a three-state model, as shown in Figure 12.7.

According to this model, the native protein (N) is in equilibrium with a partly unfolded intermediate state (I). This intermediate state may react with either another intermediate or an aggregate composed of a number of protein or peptide molecules.⁴⁸ A special kind of aggregation, fibril formation, is normally used to describe a process in which long, linear fibers are formed, whereas aggregation may result in more disordered associates. Morphological studies of protein fibril formation show the formation of different morphological species, e.g., single fibrils, helical fibrils, or bundles of fibrils during the course of fibril formation.⁴¹

Surface adsorption of proteins and peptides is characterized by the molecules' adhesion to surfaces. Because many proteins and peptides are only present in very low concentrations, the adsorption process may result in a significant depletion of drug concentrations in the formulation.



FIGURE 12.6 Chemical transformation of insulin during storage of rhombohedral insulin crystals (Bovine insulin crystals, 0.7% NaCl, 0.2% phenol) as a function of pH during storage at 25°C for 12 months. (A) Formation of the hydrolysis products monoand didesamido insulins, and the insulin split product (A8–A9). (B) Formation of covalent di- and oligomers. Adapted from Brange and Langkjaer (1993)⁴⁰ with permission.



FIGURE 12.7 Aggregation states of proteins.

Another potential problem is surface-induced aggregation in which an aggregation intermediate is formed at the surface, e.g., at the air-water or water-container interface, which initiates an aggregation or fibrillation process in the bulk.

Precipitation, which is the macroscopic equivalent to aggregation, produces a visible change of the protein or peptide solution, usually occurring as a result of denaturation.

12.4.3 STRATEGIES FOR IMPROVING STABILITY

The overall strategies for optimizing the physical stability of protein and peptide drugs include the following approaches: (i) to stabilize the native structure of the protein or peptide, for instance by promoting self-association or by maintaining native hydrophobic interaction by adding appropriate excipients/co-solvents which may stabilize through selective solvation: (ii) to prevent aggregation of partly unfolded structures, for instance by adding surfactants or co-solvents: (iii) to avoid or limit the exposure to hydrophobic surfaces, (iv) to reduce high shear forces during production; and finally (v) by chemical modification.

The simplest, yet often rather effective, excipients used to improve physical stability are salts or other ionic compounds. At low concentrations, salts can improve the stability of proteins and peptides through nonspecific electrostatic interaction. At high concentrations, however, salts may lead to destabilization depending on the protein or peptide and the nature of the salt.

It is well-known that the addition of polyalcohols and carbohydrates can stabilize proteins and peptides in a solution. At present, it is generally accepted that these compounds stabilize the protein or peptide structure by the preferential exclusion mechanism proposed by Timasheff and coworkers.^{42,49} The nature of these excipients is such that they are preferentially



FIGURE 12.8 Schematic illustration of solvent distribution in equilibrium dialysis experiments showing preferentially binding (left) and preferentially exclusion (right) to the protein. $\circ =$ water and $\bullet =$ diffusible solvent additive, e.g., sucrose. Modified from S. N. Timasheff (1992).⁴⁹

excluded from the surface of the native state of the protein or peptide, leaving the protein or peptide surface highly hydrated (Figure 12.8).

Surfactants have been shown to stabilize proteins and peptides by inhibiting aggregation and precipitation. Because of their amphiphilic character, they bind to newly exposed non-polar amino acid residues and thereby stabilize partly unfolded structures and prevent aggregation/ denaturation, since hydrophobic domains on proteins and peptides are potential denaturation sites. Moreover, surfactants also bind to interfaces, e.g., container surfaces and air-water interfaces, which are other potential denaturation sites. However, high concentrations of surfactants as well as other additives may lead to denaturation due to a radical change in the solvent environment.

Site-specific mutagenesis is another approach to improve the stability of proteins and peptides. Stability can be modified by mutating the amino acids which help to stabilize the native structure or destabilize interactions in the partly unfolded structure, or by decreasing flexibility, for instance by cross-linking via S–S bonds. However, with chemical modification there is always a risk that the therapeutic effect of the protein or peptide is reduced or even lost.

12.4.4 IMMUNOGENICITY-A POTENTIAL RISK FACTOR

Antibody formation is a potential risk factor, which should be carefully evaluated during development of protein or peptide drugs due to drug inactivation by neutralizing antibodies and the potential risk of serious side-effects, such as immunological reactions or neutralization of endogenous proteins. Although antibodies are often induced by foreign proteins, human proteins in a pharmaceutical formulation may also present a risk. For example, micelle-associated protein in epoetin formulations may present an important risk factor to the development of antibodies in patients.⁴³

12.5 ANALYTICAL METHODOLOGY

The analysis of protein formulations differs markedly from analysis of most common drug products. This is mainly because of the highly complex structure of proteins, which results in many different degradation pathways. Currently, it is not yet possible to accurately predict both structure and degradation pathways solely on the basis of the amino acid sequence. This means that a large set of analytical techniques is required, which is capable of describing the protein structure and its degradation pathways during storage at a sufficiently accurate level.

Below, many of the techniques that are commonly used in protein formulation analysis are summarized. It is beyond the scope of this chapter to go into any details, and the interested reader is referred to the book *Methods* for Structural Analysis of Protein Pharmaceuticals,⁴⁴ which describes most techniques in detail. A much more limited description can be found in reviews by Wang (1999, 2000)^{45,50} and Chen (1992).⁴⁶ The techniques which are mentioned below are summarized in Table 12.3.

12.5.1 ANALYSIS OF THE THREE-DIMENSIONAL PROTEIN STRUCTURE

The three-dimensional structure of a protein can be resolved at the atomic level using x-ray crystallography (XRC) or nuclear magnetic resonance spectroscopy (NMR). Each of these two techniques has its own advantages and limitations, but neither of them are particularly suited for protein analysis during formulation. First, these techniques are very time-consuming. Second, the samples need to be relatively pure. Third, XRC requires the protein to be able to crystallize while NMR cannot deal with proteins with a molecular weight above ca. 20–25 kDa. However, knowledge of the three-dimensional structure can be very useful for understanding the protein function as well as its physicochemical behavior in vitro. Thus, an effort should be made to elucidate the protein structure at atomic level.

In certain cases we can make qualified guesses on the protein threedimensional folding by using the ever-expanding database of known protein folds. That is, using certain computer programs we look for those proteins in a database which have a high sequence similarity to the unknown protein. It is then assumed that the folding of the unknown protein is similar to that of the known proteins with the highest sequence similarity. The structure can eventually be further optimized using other computer programs, and often this approach is quite successful. However, deviations from the actual structure occur frequently as well as the inability to model certain areas in the protein.

Determination of the three-dimensional structure using any of the techniques mentioned above requires an accurate knowledge of the amino acid sequence and the formation of intramolecular cross-links (cystine or S–S bridges). The amino acid sequence can sometimes be deduced from the nucleotide sequence of the gene involved, but there are quite often differences

due to gene-splicing and/or post-translational modification. Thus, the amino acid sequence and the disulfide bonding pattern are generally determined experimentally using techniques like Edman degradation and peptide mapping, which often involves liquid chromatography combined with mass spectrometry.

12.5.2 ANALYSIS OF PROTEIN DEGRADATION

The regulatory agencies require that a drug product is stable for up to two years. This means that protein degradation pathways need to be determined and continuously monitored during the formulation process. Any deviation in the three-dimensional protein structure can, in theory, be deleterious to its activity and safety. Thus, both the chemical and physical integrity of the protein should be determined.

The techniques for analyzing the primary structure, especially peptide mapping using liquid chromatography coupled to mass spectrometry, are usually fully capable of elucidating chemical degradation pathways. However, these methods are quite complex and laborious and often provide much more information than is required. A simple reversed-phase HPLC (RP-HPLC) method may suffice to identify and quantitate several chemical degradation processes like oxidation, deamidation, disulfide scrambling, and hydrolysis. Initially, it may be necessary to identify the various extra peaks in the chromatogram using, e.g., mass spectrometry. However, when several formulations are screened, it is only important to know which formulations reduce the chemical degradation.

Other techniques that are used to rapidly monitor chemical protein degradation are ion-exchange chromatography (IEX, deamidation and hydrolysis) and iso-electric focusing (IEF, deamidation and hydrolysis), and more recently various capillary electrophoretic methods have been introduced. Mass spectrometry can also be used as a stand-alone method; it is a very sensitive method, but not quantitative.

The analysis of the *physical* integrity of a protein is particularly challenging. XRC and NMR cannot be used for the reasons mentioned earlier. Thus, less detailed and high-throughput methods are required. For example, the global secondary structure of a protein can be determined using vibrational spectroscopy (FTIR and Raman spectroscopy) or far-UV circular dichroism (CD). Any deviation from the native state spectrum suggests partial unfolding of the protein. Vibrational spectroscopy has especially been important in the development of freeze-dried (lyophilized) formulations, since it is one of very few techniques amenable to solid samples. The presence of a native tertiary structure around certain absorption centres (such as tryptophan, tyrosine, phenylalanine and cystine) can be evaluated using near-UV CD, Raman, fluorescence, and UV spectroscopy.

Rapid methods to look at the global shape of the protein are light scattering (DLS/SLS), size-exclusion chromatography (SEC), polyacrylamide

TABLE 12.3 Analytical techniques used for the analysis of peptide and protein formulations

Technique

Structural Information

Remarks

Three-dimensional structure at atomic level	Protein must be able to form crystals; pure samples only
Three-dimensional structure at atomic level	Protein must be smaller than ca. 25 kDa; pure samples only
Three-dimensional structure at atomic level	At best an estimate, and requires comparison with other techniques
Amino acid sequence	Limited number of amino acids
Amino acid sequence; disulfide bonding pattern	Usually a combination of RP-HPLC and Mass spectrometry + various (bio)chemical methods to hydrolyze the protein at specific places in the sequence
Various chemical degradation pathways (mainly oxidation, but also deamidation, disulfide scrambling, and hydrolysis)	Often combined with mass spectrometry to determine the identity of the observed peak
Mainly deamidation and hydrolysis	Limited resolution compared to iso-electric focusing
Mainly deamidation and hydrolysis	Very high resolving power
Various chemical degradation pathways, but also (non-)covalent aggregation	
Global secondary structure; some insight into tertiary structure from Raman spectroscopy	Especially useful in lyophilization formulation development
Global secondary structure	
Some insight into tertiary structure	
	 Three-dimensional structure at atomic level Three-dimensional structure at atomic level Three-dimensional structure at atomic level Amino acid sequence Amino acid sequence; disulfide bonding pattern Various chemical degradation pathways (mainly oxidation, but also deamidation, disulfide scrambling, and hydrolysis) Mainly deamidation and hydrolysis Mainly deamidation and hydrolysis Various chemical degradation pathways, but also (non-)covalent aggregation Global secondary structure; some insight into tertiary structure Some insight into tertiary structure

Fluorescence spectroscopy UV spectroscopy

Light scattering (SLS/DLS) Size-exclusion chromatography Gel electrophoresis (PAGE) Electron microscopy (and variants) Turbidity/nephelometry Differential scanning calorimetry (solutions) Thermal unfolding

Affinity chromatography Enzyme-linked immunosorbant assay (ELISA) Surface plasmon resonance

Functional assays Karl-Fisher titration Thermogravitometric analysis Differential scanning calorimetry (solids) Powder diffraction Some insight into tertiary structure Some insight into tertiary structure; protein concentration Protein size and shape (Soluble) Aggregate formation (Soluble) Aggregate formation Protein size and shape; protein association Presence of insoluble particles Thermal stability of protein

Thermal stability of protein

Global knowledge on various degradation processes Global knowledge on various degradation processes

Global knowledge on various degradation processes; association constant Global knowledge on various degradation processes Water content of formulation Water content of formulation Glass transition temperature of matrix; crystallization of additives Crystallization of additives Very sensitive to aggregate formation

Yields various thermodynamic parameters, including unfolding enthalpy Various methods can be used as "detector" of the unfolding process; technique-dependent melting point Any degradation may result in reduced affinity Any degradation may result in reduced signal

Advanced "ELISA" method

Used in lyophilization development Used in lyophilization development Used in lyophilization development

Used in lyophilization development

gel electrophoresis (PAGE), and various electron microscopy (EM) techniques. SEC and PAGE are used to quantify the amount of soluble aggregates (noncovalent and covalent) in the solution. Light scattering is used to determine the first onset of aggregation. It is extremely sensitive, but often not quantitative. Insoluble aggregate formation is typically analyzed using turbidity or nephelometry measurements.

Other methods utilize thermal unfolding as a stability-indicating parameter: the higher the thermal midpoint for unfolding, the better the physical stability during storage. This thermal midpoint as well as other thermodynamic parameters can be determined most accurately using differentical scanning calorimetry (DSC). One can also use the various spectroscopic techniques mentioned above to plot the unfolding as a function of temperature. However, it should be noted that the unfolding midpoints can be technique dependent.

There are also global methods which only detect whether a "binding" site is still folded correctly. Such methods are affinity chromatography (AC), enzyme-linked immunosorbant assay (ELISA), surface plasmon resonance (SPR), and various functional in vitro, ex vivo or *in vivo* assays. One cannot deduce from these methods whether there has been either chemical or physical degradation, or whether the additives merely block the "active" site.

The methods mentioned above are all direct methods, i.e., they survey some part of the protein molecule directly. There are, however, also some indirect methods which can be very useful for formulation development of lyophilized products. First, the water content of a lyophilized formulation must be low to ensure long-term stability. This water content is often determined using Karl–Fischer titration or thermogravitometric analysis (TGA). Second, the product has to be stored below the glass transition temperature (T_g) , which can be determined using DSC. Finally, some additives tend to crystallize, and one must make sure that these compounds are already fully crystallized before the end of the lyophilization cycle. The presence and amount of crystalline material can be determined by means of powder diffraction or DSC.

12.5.3 PROTEIN FORMULATION AND ANALYTICAL METHODOLOGY IN PRACTICE

The approach to formulation development and the set of analytical techniques which are used can differ from lab to lab, protein to protein, and even person to person. There are a number of reasons for this, which are outlined below.

The set of techniques to be used depends very much on the stage of the formulation development. During the initial pre-formulation phase there is often a focus on one or two main degradation pathways of the protein. These main degradation pathways usually differ greatly between different proteins, and thus the required analytical methodology differs very much.

Various additives are then screened with regard to their ability to reduce these degradation pathways. Sometimes, certain additives, or the formulation as such, make the use of certain analytical techniques impossible. Thus, the chosen analytical methodology not only depends on the protein, but also on the formulation and the additives.

Only a limited number of techniques are required during the initial formulation development phase. Preferably, they should be able to rapidly screen a large number of samples. The techniques need not be quantitative but should be very sensitive, so as to be able to compare the various formulations rapidly. In the final phase of the development it is required that the degradation processes are determined more accurately in order to fine tune the formulation is stable for up to two years. The techniques used during this phase of the development need no longer be high throughput methods, but have to be quantitative.

12.6 CONCLUDING REMARKS

Protein and peptide formulation is a particularly challenging field in drug development. First, proteins and peptides are very labile drugs, and both physical and chemical degradation may result in a decreased or altered activity. Thus, the formulation should be designed to minimize this physico-chemical degradation. Second, proteins and peptides often have a low bioavailability due to their size as well as their rapid elimination *in vivo*. This means that these drug compounds usually have to be administered parenterally, which, in turn, puts additional constraints on the formulation characteristics. The formulation scientist therefore has to balance on a very thin rope, continuously having to try and improve the physicochemical stability and shelf-life of the product, while maintaining the suitability of the formulation for parenteral injection.

The problematic delivery of proteins and peptides has further increased the interest in alternative delivery strategies. These alternatives range from chemical modification of the drug compound to alternative routes of administration, and from peptidomimetics to sustained and controlled release systems.

In the last decade much progress has been made as regards to understanding how to improve the physicochemical stability of proteins and peptides as well as the effect of various excipients in the formulation. A large and still expanding set of analytical techniques have been developed for this purpose. As a result, proteins and peptides have become an important and major class of drug products, despite their low stability and bioavailability. At present, about one-third of every new drug product contains a protein or peptide as its active compound, and this percentage is expected to rise in the near future. The next decade will therefore be an exciting time for those with an interest in protein and peptide formulation.

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13 Micro- and Nanoparticulate Polymeric Delivery Systems for Nucleic Acid-Based Medicine

Shikha P. Barman and Mary Lynne Hedley

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13.1 INTRODUCTION

Therapies based on nucleic acid technologies have the potential to treat a variety of diseases, by mechanisms as diverse as transgenic expression of proteins relevant to genetic deficiencies, in the elucidation of potent immune responses relevant to chronic viral disease, cancer or allergy, or in the down-regulation of specific disease causing proteins. Hindering the fast-paced development of these therapies are issues associated with the short half-life of nucleic acid *in vivo*, inefficient cellular internalization, degradation in acidic endolysosomal vesicles and inefficient transport through the nuclear membrane.¹ Given these hurdles, it is not surprising that results from clinical studies utilizing "naked" unformulated DNA have proven disappointing. Viruses, as gene delivery carriers, have demonstrated higher transgene expression in clinical studies, but issues of toxicity, tolerability and cost continue to drive the search for non-viral nucleic acid delivery systems.

The focus on particulate polymeric delivery systems centers on their ability to protect DNA from interstitial nucleases and target DNA for cell uptake. Macrophages and cells of the reticuloendothelial system (RES) phagocytose particles less than $10 \,\mu\text{m}$ in size, whereas many cell types internalize particles less than $1 \,\mu\text{m}$ by the process of pinocytosis. To enhance or direct cellular uptake, modifications that encourage thermodynamically favorable particle:cell interactions such as the addition of cationic charges or receptor-targeted surface ligands can be incorporated into DNA-particulates. Also integral to certain of these formulations are endosomolytic agents and pH-stimulated cationic molecules that enable rapid rupture of the endolysosomal membrane and release of DNA into the cytoplasm.

Polymers useful for DNA encapsulation can be either hydrophobic, such as poly(L-glycolic acid) (PLGA), poly(L-lactic acid) (PLA), poly(D,L-lactideco-glycolide) (PLG), copolymers of fumaric acid and sebacic acid (poly-(FA:SA)), and poly(alkylcyanoacrylates) or hydrophilic, such as poly(ethylene glycol) (PEG), chitosan, gelatin, and albumin. A variety of manufacturing methods including solvent evaporation/extraction, complex coacervation, interfacial/monomer/emulsion polymerization, thermal denaturation and cross-linking and spray drying have been developed to incorporate nucleic acids within micro- or nanoparticles, each with its own technical challenges. Regardless of the method used for encapsulation or association of particles with nucleic acid, there is a need to achieve high loading so as to minimize patient dosing, control release and thus drug availability, to protect DNA from degradation in the endosomal compartment following cellular uptake, and to enhance the release of nucleic acid from these vesicles so as to increase drug potency. This chapter focuses on general methods used for production of particulate nucleic acid delivery systems, and the steps researchers have taken to meet the need for a high-loading, potent, DNA- particle formulation.

There has been significant progress towards the therapeutic use of polymeric particulate based nucleic acid delivery systems. Results from multiple clinical trials appear to be quite interesting and promising. At least one formulation has been tested in a randomized, placebo controlled late-stage clinical study and demonstrated remarkable efficacy in a select patient population. The results of this and other studies will also be discussed within this chapter.

13.2 METHODS OF PREPARING ENCAPSULATED NUCLEIC ACID

13.2.1 SOLVENT EVAPORATION/EXTRACTION

Solvent evaporation/extraction methods are useful for encapsulating nucleic acid in hydrophobic polymers such as PLGA, PLA, PLGA–PEG and other related co-polymers. The method requires the use of emulsions (e.g., water–oil–water (W/O/W) or water–oil–oil (W/O/O)) to entrap an aqueous phase containing DNA in a water insoluble polymer.

13.2.1.1 Emulsions

An emulsion is defined as the dispersion of one liquid phase (dispersed phase) in another immiscible liquid phase (continuous phase), the dispersion being stabilized by an amphiphilic compound called an emulsifier. Emulsions in which water is the internal dispersed phase and an organic solvent is the continuous phase are termed water-in-oil emulsions (W/O; Figure 13.1); whereas, emulsions in which oil is the dispersed phase and water is the continuous phase are termed oil-in-water emulsions (O/W; Figure 13.2). Emulsions are created by mechanical agitation of two immiscible liquids, the energy of agitation causing dispersion of one liquid in another, forming droplets that are subsequently broken up into smaller ones with continued input energy. The energy of agitation helps to overcome viscous pressure gradients exerted by the cohesive forces of the individual liquids. The maximum droplet diameter that can be obtained with a given kinematic viscosity of the continuous phase is inversely proportional to the energy of agitation; smaller droplets are formed with higher frequencies of agitation. Increasing the viscosity of the continuous phase can overcome the cohesive resistive forces exerted by the dispersed liquid phase, resulting in smaller droplets when mixed at a fixed frequency of agitation. Conversely, increasing the viscosity of the dispersed phase results in enhanced resistive, cohesive forces toward droplet formation, resulting in larger droplets, provided all other parameters including the energy of agitation and outer phase viscosity

1. Create Primary Emulsion

Aqueous nucleic acid solution is added to polymer-containing organic phase.



The particles are washed with water to remove excess emulsifier and solvent. The concentrated particles are lyophilized.

FIGURE 13.1 Schematic of a W/O/W microparticle preparation process.

are held constant.² Droplets formed in emulsions are thermodynamically unstable, and favor coalescence, due to the high surface tensive energy. Addition of an emulsifier or a surface-active agent to the continuous phase lowers the interfacial surface tensive energy by adsorbing to the droplets as a monolayer at the water–oil interface. Emulsifiers are amphiphilic, with both hydrophobic and lipophilic components. The type of emulsion formed (W/O or O/W) depends upon the hydrophilic/lipophilic properties of the emulsifier. Surface-active agents can be assigned a numerical value representative of their hydrophilic/lipophilic properties. For surfactants such as a fatty acid, (e.g., sodium lauryl sulfate), hydrophilic: lipophilic balance (HLB) value is defined as HLB = 20(1-S/A), where S represents the saponification number of the ester and A the number of acid groups. For nonionic surfactants such as polyethylene glycol, the relationship HLB = (E + P)/5 is appropriate, where E represents the weight percent of the oxyethylene and P is the weight percent of

1. Create Primary Emulsion

Aqueous nucleic acid solution is added to polymer-containing organic phase.



FIGURE 13.2 Schematic of a W/O/O microparticle preparation process.

polyhydric alcohol. HLB values are usually available from the manufacturer. Amphiphiles with low HLB (3–6) stabilize W/O emulsions, while high HLB surfactants (9–12 and higher) stabilize O/W emulsions. Therefore, a blend of Tween 20/Span 20 (HLB \sim 12) will support an O/W emulsion.^{2,3}

When an initial emulsion is added to a second continuous phase, a complex emulsion such as a W/O/W emulsion is formed. An emulsifier is used to stabilize the emulsion, and, the stability of the second emulsion can be attributed to the strength of the interfacial film formed by the emulsifier on the droplets, the strength of the film being dependent upon the structural compatibility of the hydrophobic and hydrophilic components of the emulsifier (HLB) to that of the organic (O) and aqueous (W) phases.

13.2.1.2 W/O/W Emulsions

Dispersion of an aqueous solution of nucleic acid within a solution of water-insoluble polymer (e.g., PLG, PLA) in an organic solvent such as

dichloromethane, ethyl acetate or chloroform creates a W/O emulsion. The emulsification process requires input energy usually supplied by homogenization, or sonication of the W/O mixture to produce aqueous droplets of a given size (primary emulsion). The primary W/O emulsion is added with agitation to a second aqueous phase containing an emulsifier. The insolubility of the polymer in water causes micro-collapse of the polymer chains toward partial precipitation and creates a turbid, colloidal emulsion. Subsequent to the formation of the secondary emulsion, the solvent is removed from the system (Figure 13.1). Depending upon the vapor pressure of the organic solvent at the temperature of stirring, its saturated solubility in the continuous aqueous phase, and the relative volumes of each phase, the mechanism of solvent removal can be extraction, or evaporation.^{4,5} Evaporation of the organic solvent by heating, or extraction by dispersion of the emulsion in a large volume of aqueous phase, results in particles containing nucleic acids. The particles can be concentrated and lyophilized to produce a free-flowing powder.

Particle size is easily manipulated in the W/O/W process and particles that range from < 1 to $> 100 \,\mu\text{m}$ can be generated. Factors that contribute to particle size include the input energy required for droplet formation^{5–8}, and the viscosity of the aqueous continuous phase^{8–10} a property altered by increasing the concentration, or molecular weight of the emulsifier in the continuous phase. For example, the use of poly(vinyl alcohol) (PVA) or poly(vinyl pyrrolidone) (PVP) at high concentrations or high molecular weight will result in smaller particulate sizes, relative to low concentrations and low molecular weight compounds.

The internal morphologies of particles produced by the W/O/W method have matrix, honeycomb, vacuole-rich, or hollow characteristics. Organic solvent volatility and solubility in the aqueous, continuous medium plays a large role in determining the resulting internal morphology of a particle prepared by the W/O/W process. Solvent removal by evaporation is relatively slow due to the low solubility of organic solvents in water. As solvent leaves the nascent particles, vacuoles are formed within the semisolid droplet to produce a particle with honey-combed internal morphology. Factors that act to more rapidly remove organic solvent from nascent particles will produce more vacuole-rich and hollow like particles. The use of an organic solvent with high water solubility is one way to increase the rate of solvent extraction from a W/O/W system and produce thin-walled particles.⁴ At increased concentrations of the emulsifier polyvinyl alcohol (PVA), the solubility of organic solvents in the secondary aqueous phase is enhanced so that solvent is rapidly extracted from the nascent particles producing an internal hollow morphology.⁴

The W/O/W process has been used successfully to encapsulate nucleic acids and numerous studies have now demonstrated that encapsulated plasmid DNA is protected from nuclease digestion.^{10–12} Gene expression vectors encapsulated in particles produced by the W/O/W process are bioactive when injected into test animals and elicit desired therapeutic responses whether

delivered orally $^{13-16}$ or parenterally 17,18 including protection in tumor models. 18

Of issue when encapsulating plasmid vectors by the W/O/W technique is the need to preserve DNA structural integrity during formation of the particles. Degradation from the supercoiled to less stable nicked and linear isoforms occurs as a result of the high-energy microcavitation created during physical methods of emulsification (by homogenization sonication).^{19,20} DNA supercoiling is preserved if the heat generated during primary emulsification is dissipated. Cooling the emulsion or using a cold, semi-frozen solution of DNA during primary emulsification has been reported to preserve plasmid supercoiling.²¹ DNA can also be protected by organic solvents such as methylene chloride ethyl acetate, ethyl formate and chloroform^{8,12,21} and stabilizing DNA during homogenization/sonication by the addition of pH controlling buffers,²¹ or agents such as EDTA.^{8,21,22} The process of lyophilization, or slow freezedrying, induces crystallization of buffer salts present in the formulation. Buffer crystals, being in close proximity with DNA, cause distortion of the DNA structure, resulting in loss of supercoiling. Crystal formation can be prevented by incorporation of saccharides such as mannose, sucrose or dextrose in the primary and secondary emulsion.²¹

A second key challenge associated with the double emulsion method is achieving high efficiency of encapsulation. An osmotic gradient, or difference in chemical potential between the inner dispersed phase and outer continuous phase causes movement of the highly hydrophilic DNA from the hydrophobic droplet, into the secondary aqueous phase. If a surfactant is associated with the microparticle, loss of DNA may be further exacerbated by diffusion of DNA-associated surfactant to the secondary phase.^{8,10} Osmotic gradients can be reduced or eliminated and encapsulation efficiency enhanced by the addition of sodium chloride, calcium chloride or magnesium chloride, to the external aqueous phase.^{23–25}

The aqueous mobility of DNA is reduced by condensation with cationic polymers or lipids, which neutralizes DNA charges and reduce water solubility.^{10,11,26,27}. Micronized nucleic acid is efficiently encapsulated due to its reduced mobility in comparison to DNA in aqueous solution.²⁸ Nucleic acid/polymer/lipid, and DNA/quaternary ammonium salt complexes have reduced water solubility and may promote association of nucleic acid with the polymer to enhance encapsulation efficiency.²⁹ The mobility and solubility of DNA can be reduced by decreasing process temperatures,²¹ and by increasing the overall density, viscosity or hydrophobicity of the organic droplet that further impedes the diffusion of DNA to the outer aqueous phase. For example, the use of high molecular weight polymers (e.g., poly(d,l-lactide co-glycolide) (PLGA) of 30,000 vs. 12,000) in the W/O/W process increase DNA loading.4 Conversely, the use of hydrophilic poly(L-glycolic acid) (PLG) polymers with uncapped end-groups, decreases the encapsulation efficiency relative to that achieved with capped end-groups.¹⁰

13.2.1.3 W/O/O Emulsion-Phase Inversion

A second type of emulsion-based method for making microparticles containing nucleic acid, W/O/O, differs from the W/O/W technique by the exchange of the secondary aqueous phase for an organic (O) phase. For the process to be effective and the polymer must be highly soluble in the first organic solvent, highly insoluble in the second organic solvent. In addition, the first and second organic phases must be highly miscible and the second organic phase must be miscible with water. The primary W/O emulsion is formed in much the same manner as in the W/O/W method, and the secondary emulsion is formed by the addition, with mixing, of a second organic solution. As the aqueous phase and the first organic phase diffuse into the secondary organic phase, the polymer chains undergo a "phase inversion" to create solid particles (Figure 13.2). The rate of extraction of the first organic phase into the second organic must not be so fast, as to cause disintegration of the polymer particles into a precipitate, but high enough to cause solid particle formation from the droplets in the primary emulsion. The particles can be washed with water to remove residual organics and lyophilized to produce a free-flowing powder.³⁰ This method avoids the loss of DNA into a secondary aqueous phase and leads to enhanced encapsulation efficiency.30

Plasmid DNA is encapsulated by the phase inversion technique in copolymers of fumaric and sebacic anhydride (FA:SA) using methylene chloride and petroleum ether as the first and second mutually miscible organic solvents. Plasmid DNA, suspended in an aqueous solution of lecithin is emulsified with a polymer-methylene chloride solution. The emulsion is lyophilized to remove water, and re-solubilized in methylene chloride to produce a DNA-in-polymer suspension (O) that is introduced into a bath of petroleum ether (O) resulting in particle formation by phase inversion. DNA encapsulation efficiency is between 75 and 95%, and plasmid integrity is maintained. Co-encapsulation of DNA and chloroquine by this technique produces particles that mediate transgene expression in transfected cells.^{31,32}

In a further modification of the phase inversion method, cetyldimethylammonium bromide (CDAB), a cationic, highly hydrophobic molecule, dissolved in a mixture of chloroform and ethanol, is added to an aqueous solution of plasmid DNA to promote complexation by charge neutralization. The organic phase containing CDAB:DNA is mixed with a second organic phase comprised of PLG dissolved in acetone. The resultant organic phase is emulsified with PVA to form particles by phase inversion. The choice of solvents (balance of polar and non-polar) and enhanced DNA hydrophobicity imparted by association with CDAB, enable dissolution or partitioning of nucleic acids into the organic phase. The advantage to this method is that the efficiency of extracting DNA into the organic phase is $\sim 99\%$; encapsulation efficiency approximated 76%. Furthermore, the integrity of the plasmid was maintained as this process avoided high-shear homogenization or sonication, commonly used to prepare emulsions. Activity of particulate DNA formed in this manner has not yet been reported.²⁹ Overall, the use of a double emulsion method to entrap nucleic acids is extremely versatile in allowing the manipulation of several parameters to enable optimization of loading, particle size, and nucleic acid release. The system is also amenable to production of particles that can be coated with molecules such as cations and cell targeting moieties by addition of these factors to the secondary aqueous phase or the final wash buffer prior to lyophilization.

13.2.2 COMPLEX COACERVATION

Complex coacervation is a process by which ion-pair interaction and subsequent charge neutralization ("complexation") between two oppositely charged polymeric colloids in an aqueous solution result in phase separation ("coacervation") and particle formation. Historically, drug-containing particles prepared in this manner contain naturally occurring polymers such as cationic gelatin coacervated with anionic gum arabic. Complex coacervation is induced by the addition of salts to ionic polymers. This leads to collapse of the polymer chains into reduced hydrodynamic volumes and the formation of nanoparticulate precipitates. With continued addition of salt, the coacervates eventually phase-separate out of solution. The un-reacted components can be removed for example, by centrifugation in a sucrose step gradient and by dialysis (Figure 13.3). Depending upon the strength of the ionic interaction between the two oppositely charged electrolytes and the stability of the coacervate at neutral and low pH, particles produced in this manner may require chemical cross-linking. Particles are washed, concentrated and dried for storage.

Nucleic acids are an ideal candidate for encapsulation by this method given their polymeric, anionic nature. Nucleic acid-containing particles prepared by complex coacervation are nano- or micro-particulates, depending upon the concentrations of the participant polymers.^{33,34} The particles are spherical and consist of a solid core with nucleic acid embedded in a polymer matrix.³⁵ Nanoparticles of chitosan-DNA are prepared by complex coacervation with sodium sulfate as a desolvating agent. Charge interaction between chitosan and DNA is strong, regardless of the pH, therefore, no crosslinking of the particles is required.^{33,36–38} For gelatin/DNA systems, the complexes are stabilized with persulfate-based crosslinkers.^{35,38,39,40} Partial in vitro protection from nucleases and *in vivo* bioactivity has been demonstrated for chitosan/DNA and gelatin/DNA nanoparticulates;^{35,38,39,41} including the ability to protect mice from a viral^{37,40} or allergen³⁶ challenge when used to deliver a DNA encoded antigens.

Factors that affect the kinetics and thermodynamics of phase separation include pH, temperature, salt and polymer concentration, stirring rate, and reactor dimensions.³⁵ The pH of the reaction medium must be selected such that the nucleic acid is stable and at the same time both of the oppositely charged polymers are in an ionized state. Complete ionization of all charged groups ensures efficient mutual binding of the polymers before

I. Complexation and Coacervation

DNA and water soluble cationic polymer are complexed in aqueous solution. Addition of a desolvating agent forms particulate coacervates.



II. Crosslinking

If necessary, particles are cross-linked by a chemical reaction



III. Purification and Drying

Particles are dialyzed to remove cross-linker and lyophilized.



FIGURE 13.3 Schematic of a complex coacervation process for preparing microparticles.

phase-separation. Care must be taken to control temperature during complex coacervation since phase separation at high temperatures causes rapid coacervation and random chain-entanglement that results in coagulation and aggregation. Optimal nucleic acid and cationic polymer concentrations can be determined stoichiometrically, based on the number of charged moieties on each polymer. Typically, the nucleic acid is utilized as the rate-limiting polymer so as to drive complete encapsulation.

The most significant advantage of this system is the high efficiency with which nucleic acid is entrapped in particles; ~98% of input DNA is encapsulated. Agents that impart functional attributes to the delivery system can be co-encapsulated with the nucleic acid or conjugated to the microparticles surface. Examples of these are chloroquine, transferrin or PEG, co-encapsulated or conjugated to microparticles surface within chitosan-DNA or gelatin-DNA coacervates so as to impart specific functionalities, such as enhanced endosomolytic activity, specific cell targeting or extended *in vivo* circulation.^{33,41} The significant disadvantage to this process lies in its exquisite
sensitivity to process variables and, in some cases, the need for chemical crosslinking post particle formation; factors which influence the robustness of the process and the ease of manufacturing scale-up.

13.2.3 THERMAL DENATURATION AND CROSSLINKING

A protein, such as albumin, dissolved in aqueous solutions, is coacervated into nanoparticulates, by gradual addition of a desolvating agent (e.g., ethanol). The desolvation, indicated by solution turbidity, is gradual and dependent upon the volume of desolvating agent relative to the volume of the protein solution and the solution temperature.⁴² Post-desolvation, the nanoparticles are stabilized by cross-linking with amine crosslinkers (e.g., glutaraldehyde, formaldehyde). Alternatively, particle stabilization is accomplished by thermal denaturation (Figure 13.4).⁴² Gelatin nanoparticulates

1. Desolvation

Protein in aqueous solution precipitates upon the addition of a desolvating agent to form protein particles



2. Cross-linking/ Thermal Denaturation

Particles are cross-linked by chemical and/or thermal reaction.



3. Purification

Particles are dialyzed to remove cross-linker.

Dialysis



4. DNA Adsorption and Lyophilization

Particles are coated with DNA and lyophilized.



FIGURE 13.4 Schematic of a process to prepare microparticles by thermal denaturation/crosslinking.

are prepared in a similar manner, using an acetone-water desolvating system followed by stabilization with either glutaldehyde crosslinking or heat denaturation.

Particles of gelatin or albumin, formed in this way, range from 20 to 400 nm depending upon the volume of desolvating agent added. Twenty to fifty (20-50) nm albumin particles are obtained with 0.75 ml ethanol/ml albumin; whereas, 400 nm particles are obtained when using 2.0 to 3.0 ml ethanol/ml albumin. Particle size reaches an equilibrium value after a certain amount of ethanol addition, increasing only in particle concentration beyond this point.⁴²

The surface charge of these particles is cationic, due to the presence of amine groups on the proteins and thus, nanoparticles obtained by this process are amenable to surface-adsorption of nucleic acid to form coated particles. Association of oligonucleotide (ODN) with albumin particles can protect the nucleic acid from degradation and enhance delivery to the nucleus of cells.⁴³ Furthermore, other targeting moieties can be adsorbed to the cationic surface if nucleic acid delivery to specific cells is desired. For example, carboxylates present in albumin are covalently conjugated with polyethyleneimine (PEI). The conjugation reaction creates nanoparticulate aggregates of albumin/PEI, which are thermally denatured at 85°C to stabilize the particles. Incubation of plasmid DNA with the albumin/PEI particles; leads to efficient adsorption of DNA.⁴⁴ The particles display enhanced particle : cell interaction and transfection in vitro, and *in vivo* the particles selectively distribute to the lung tissue following intravenous injection and promote transgene expression.⁴⁴

13.2.4 SPRAY-DRYING

Encapsulated DNA is produced by spray-drying, a combination of emulsifica-tion and air-drying techniques.²² The process involves three basic steps: (a) nebulization of plasmid DNA emulsified in a polymer-organic solvent solution (e.g., ethyl formate, ethyl acetate, methylene chloride, chloroform), (b) warm air-drying of the aerosolized polymer/DNA-containing droplets and (c) collection of the dried aerosolized product by separation of the dried product from the solvent-rich vapor phase (Figure 13.5). Alternatively, nucleic acid particulates are dispersed in the polymer-containing organic phase, then subsequently, nebulized and air-dried. The internal diameter of the atomizer is generally between 0.5-1 µm, producing fine, spherical droplets, less than 10 µm in diameter. The size of the particles is directly correlated to the flow rate of the atomizer and the viscosity of the polymer solution, which is controlled by solution temperature, polymer molecular weight and concentration. High viscosity solutions are not easily sheared into atomized droplets using spray nebulizers and result in filaments rather than microparticles.⁴⁵ The flow rate of liquid is typically between 15 and 20 mL/min. The temperature of the drying chamber can be between 40 and 200°C, allowing the choice of an aqueous or organic-based system.

1. Formation of a primary emulsion

Aqueous nucleic acid solution is added to polymer-containing organic phase.



4. Collection

Dried microparticles are channeled into a collection chamber.

FIGURE 13.5 Schematic of a process to prepare microparticles by spray drying.

Encapsulation efficiencies are high with this process as loss of DNA to a secondary aqueous phase is eliminated; plasmid structural integrity is preserved, and biological activity maintained.^{22,46,47}

The primary advantage to this system for nucleic acid/particle production is its amenability to scale-up. Since the process is continuous, processing times can be increased if larger batches of material are desirable. Particle manufacturing with spray-drying is reproducible, suitable for aseptic processing, and is cost-effective rendering this method highly attractive for the large scale production of nucleic acid-containing polymer particulates for parenteral delivery. An additional advantage of this method is that the technology platform is applicable to several types of polymers (e.g., PLG, PLA, poly(hydroxybutyrates), poly(caprolactones), carboxymethyl celluloses) and/ or excipients of various hydrophobicities and molecular weights (glucose, albumin, mannitol). The major disadvantage of spray drying technique is that the high temperature may affect DNA stability.

13.2.5 INTERFACIAL/MONOMER/EMULSION POLYMERIZATION

Particle formation by interfacial emulsion polymerization, involves emulsification of monomers, *n*-vinyl alkyl cyanoacrylate (alkyl: *n*-isobutyl-, *n*-isohexyl-, and *n*-hexyl), with a water soluble initiator to promote polymerization within micelles. The components of this emulsion system include a water-insoluble liquid monomer, an amphiphilic emulsifier, and a water-soluble initiator (e.g., ammonium persulfate) dissolved in an aqueous medium. Post-emulsification, the system is comprised of micelles containing the hydrophobic monomer, with the initiator residing in the continuous, aqueous phase (O/W). Polymerization of the monomer occurs very quickly after micelle formation and is initiated at the oil/water micellar interface and chain-propagated to create the polymer particulates (e.g., poly(alkylcyanoacrylates) (PACA) (Figure 13.6). The rate of polymerization is controlled by the pH of the aqueous medium, which must



FIGURE 13.6 Schematic of a process to prepare microparticles by interfacial monomer/ emulsion polymerization. be less than pH 2.5, to prevent rapid formation of an insoluble, tangled, polymer colloid. After polymerization is complete, the pH of the suspension is neutralized, larger aggregates are removed (e.g., by centrifugation), and the particle suspension is dialyzed to remove salts and residual monomer. The nanoparticles are stored in an aqueous suspension or dried to produce a powder. Particles obtained by this method are in the submicron range, the actual size of the micellar droplets being controlled by the viscosity of the aqueous medium and the energy of agitation, as for any emulsion. Nucleic acids must be adsorbed to these particles due to DNA instability in the low pH polymerization medium.

An advantage in using this method of particle preparation is that particles are preformed and the manufacturing process need not be amenable to nucleic acids. In addition, these polymers are biodegradable, biocompatible and have a history of safe use as bioadhesives in humans.⁴⁸ PACA particles produced by this method have been studied extensively in the delivery of oligonucleotides.^{49–52} PACA-ODN formulations penetrate cells more readily than ODN alone and as a consequence improve the efficiency of ODN activity *in vivo*.⁴⁹

13.3 PRE-FORMED PARTICLES

13.3.1 Adsorption of Nucleic Acid

As previous examples have highlighted, an alternative to encapsulating DNA within a polymeric particle is adsorption to the external surface. Particles, pre-formed by an appropriate technique, are subsequently incubated with the nucleic acid for surface-adsorption. To enhance particle binding of the anionic nucleic acid, cationic hydrophobic compounds (e.g., diethylaminoethyl dextran (DEAE-dextran); hexadecyltrimethylammonium bromide (CTAB), polyethylenimine (PEI)) are either incorporated within the particulate matrix or adsorbed to the particle to impart a positively charged surface that is amenable for ion-pair binding (Figure 13.4 and Figure 13.6).

Poly(alkylcyanoacrylate) nanoparticles prepared by interfacial emulsion/ micellar polymerization coated with a cationic compound (e.g., CTAB, DEAE-Dextran) efficiently adsorb ODN. The efficiency of ODN binding to the cationic surface of such particles is nearly quantitative and produces a stable formulation that affords the nucleic acid protection from nuclease mediated degradation while most free unmodified ODN degraded within 5 min in serum.^{50,53,54} Studies with variety of cell lines demonstrated that the cellular uptake of (poly(isobutylcyanoacrylate) (PIBCA) adsorbed ODN was significantly greater than uptake of free ODN,^{50,51} and the activity of (poly(isohexylcyanoacrylate) (PIHCA) particle-bound ODN was detected at a 100-fold lower concentration than free ODN.⁴⁹

Plasmid DNA adsorbed to CTAB or PEI-modified PLG microparticles, is afforded some level of protection from nuclease mediated destruction, is

biologically active in transfection systems, and in the case of CTAB coated particles, elicits biological effects in animal systems^{55–58} including inhibition of tumor growth.⁵⁹ Plasmid adsorbed on PEI-modified PLG/poly(carbobenzoxy) lysine) microparticles successfully transfected cultured cells leading to transgene expression.⁶⁰

A recognized advantage of adsorbing vs. encapsulating DNA is that adsorption is quantitative and little loss of input DNA is observed.^{56,57} Moreover, DNA is not subjected to any of the shear forces associated with certain encapsulation processes.

13.3.2 COMPLEX FORMATION

In addition to the particle formulations mentioned in previous sections, surface-adsorption of nucleic acids to particles formed by complexation or selfassembly have also been tested as potential nucleic acid delivery systems. Micellar formulations are created by adsorption of plasmid DNA onto micelles formed by a PLG-graft-poly(L-lysine) copolymer. Bound DNA is protected from nucleases and exhibits high transfection efficiency.^{61,62} Micelles formed of PLG chemically conjugated to amine-terminated oligonucleotides demonstrate sustained release of ODN accompanied by gradual degradation of the PLG backbone.⁶³ Ionotropically gelled alginate microparticles adsorb ODN and the composition enhances the bioavailability of ODN in a dog model.⁶⁴

13.4 STRATEGIES TO ENHANCE CELLULAR UPTAKE

Although certain nucleic acid molecules may have a desired endosomal activity, in general, the cytoplasmic (antisense, silencing RNA) or nuclear (gene expression) activity of polymeric DNA-particulate formulations will largely depend on their uptake by cells and release from endosomal compartments. Cellular uptake requires that nucleic acid cross the plasma membrane directly, or be internalized by a process of endocytosis; both events require prolonged association between the particle and the cell. The plasma membrane is a highly complex, negatively charged, organized structure comprised of lipids, surface-associated sugar residues, sialylated glycoproteins and proteins. Repelling London dispersion forces between the anionic cell membrane and the anionically charged nucleic acids render entry of DNA thermodynamically unfavorable. Given the significance of this obstacle, it was quite surprising that unformulated "naked" DNA was taken up and expressed following injection into murine muscle.⁶⁵ Recently it was discovered that injected naked DNA is internalized as a consequence of the hydrodynamic pressure, induced by administration of a volume nearly equal to that of the injected muscle. This prolongs contact between the DNA and myocytes.⁶⁶ Since this approach is not feasible in humans nucleic acid-containing carriers that enhance DNA-cell interaction are required to create successful therapeutic DNA formulations.

13.4.1 TARGETED ENDOCYTOSIS BY PARTICLE SIZE

Certain cells such as those of the RES (e.g., macrophages, Kuppfer cells, monocytes), neutrophils, and dendritic cells have a natural tendency to internalize, or phagocytose, particles, into endosomal vesicles.^{46,55,67} These cells ingest particles below 10 μ m in diameter, and more preferably those between 1 and 2 μ m. Particle uptake by certain of these cells (e.g., dendritic cells, macrophages) is crucial to immune based therapy where the cells serve as professional activators of the humoral and cellular immune response. However, for other therapeutic approaches, it may be more desirable to avoid uptake by phagocytes and specifically target DNA-particles to other cells. Nonphagocytic cells, efficiently internalize nanoparticles. For example, in a rat in situ intestinal loop model, the efficiency of uptake for nanoparticles of size ~ 100 nm is greater than those of size between 1 and 10 μ m.

13.4.2 TARGETED ENDOCYTOSIS BY SURFACE MODIFICATION

To promote targeting of DNA-loaded particles to specific cell populations, particles are surface-modified to include compounds that bind cell surface receptors expressed by the cell of interest. Asialoglycoprotein receptors (ASGP) are found predominantly on the surface of hepatocytes and carriers for liver targeted DNA can be engineered to contain moieties that bind to these receptors. Plasmid DNA encapsulated in nanoparticles prepared from transferrin conjugated poly(aminopoly(ethylene glycol)cyanoacrylate-co-hexadecyl cyanocrylate) target tumor cells that express high levels of transferrin receptor.⁶⁹ Transferrin-conjugated DNA-chitosan and gelatin-DNA complex coacervate nanoparticles enhance transfection of transferrin receptor positive cells.^{33,35}

13.5 STRATEGIES TO ENHANCE ENDOSOMAL RELEASE

Cell internalization by endocytosis localizes particle-DNA formulations in endosomal vesicles that have an internal acidic pH. Endosomal escape is a ratelimiting step for DNA delivery given that nucleic acid is highly prone to acid mediated degradation. This effect is minimized if factors in the formulation promote rapid release of the DNA from endosomes. Endosomal rupture is mediated by destabilization of the vesicular membrane, which can occur by the interaction of compounds with the bilayer, or by buildup of hydrodynamic pressure within the vesicel.

To promote endosomal rupture of the vesicular membrane, DNA is encapsulated, or complexed with polycations. These polymers, by virtue of their primary amino groups, become protonated and highly charged at acidic pHs, incorporating H^+ ions from the acidic environment (NH_3^+), thus acting as "proton sponges." Repulsive interactions between protonated groups increase the hydrodynamic volumes of the polyplexes, which, in theory, ultimately results in physical engorgement and rupture of the endosomal membrane. Additionally, it has been suggested that buffering, or neutralization of the acidity of the endolysosome promotes swelling of the endosomal vesicle and is membrane destabilizing. Polymers minimally charged at physiological pH, but fully protonated at acidic pHs have the potential to induce membrane destabilization. For example, protonation of polymers with carboxylic acid groups (e.g., poly(propylacrylic acid)(PPAA)), triggers destabilization of lipid membranes at pH less than 6.5.^{70,71} This polymer exhibits minimum lytic activity at physiological pH, which increases to a maximum at pH 6.4, thereby providing selective membrane lysis without generalized toxicity to all cells.⁷² PPAA significantly enhances transfection *in vivo*.⁷¹

It is hypothesized that PLG itself destabilizes endosomal membranes. Hydronium ions (protons) are transferred from the acidic environment of the endosomal vesicle to PLG on the surface of microparticles, due to selective protonation of the carboxylates and hydroxyls.⁷³ Cationization of PLG particles within the endosomal compartment results in pH buffering, which promotes destabilization of the endosomal membrane, leading to release of the internalized particles.^{74,75} Delivery of nucleic acid/complexes encapsulated in PLG demonstrated preferential localization in the nucleus, an indication of successful endosomal release and nuclear transport.⁷⁶

13.6 STRATEGIES TO CONTROL DNA RELEASE

Nucleic acid must be released from particles to achieve a desired biological effect. Particles can be designed to release DNA in the cell following internalization, or in the interstitial space. DNA is rapidly degraded in the interstitium, so that if DNA is released to this location, steps must be taken to protect it from degradation.¹ The in vitro assays used to measure release of DNA from particles are not necessarily correlative with the *in vivo* release rate; however, data from release assays are useful for comparison of different formulations. A typical in vitro release experiment requires the suspension of particles in a buffer that maintains a desired and stable pH throughout the experiment, and incubation at 37°C with agitation or rotation. Temperature and buffer pH are important factors in controlling the rate of polymer degradation, which for PLG is accelerated under extreme conditions of low or high pH and elevated temperature.^{7,77} At each pre-selected time point, a volume of the supernatant is retrieved and analyzed for nucleic acid content and the release rate is calculated by determining the amount of DNA released over time.⁷⁸

The factors controlling release from particles with encapsulated vs. adsorbed nucleic acid are different. Due to the large hydrodynamic volume and size of DNA, release of encapsulated DNA is primarily controlled by the rate of polymer degradation; whereas, release of surface-adsorbed DNA is controlled by diffusion or surface erosion. Factors influencing the release rate of encapsulated DNA are (1) particle surface area, (2) porosity, (3) nucleic

acid loading (4) particle morphology, (5) polymer hydrophobicity, (6) nucleic acid hydrophobicity, and (7) excipients used during particle formation. In general, increasing particle surface area, porosity and nucleic acid loading all increase the rate of release from hydrolysis sensitive polymers.^{7,79} Each of these parameters influence release by increasing the rate of polymer solvation, hydrolysis, and degradation.

Small and porous microparticles have a large surface that promotes solvation; particles with high DNA loadings absorb water by virtue of the increase in hydrophilic nucleic acid content. Particles that are hollow or contain vacuoles release DNA more quickly than matrix-like particles made of the same polymer. The amount of internalized DNA near the particle surface affects the amount of DNA released soon after hydration. DNA-containing PLG microparticles prepared by a W/O/W emulsion-extraction method can be hollow and thin-shelled, leading to a rapid rate of DNA release,⁴ in comparison to those prepared by the W/O/W emulsion-evaporation method, where the internal microstructure of the particles ranges from hollow to vacuole rich to porous.^{5,80} Similarly, poly(lactide)-ODN particles prepared by the W/O/W emulsion-extraction method.⁵

Scanning electron microscopy (SEM) micrographs of freeze-fractured microparticles show that particles made with DNA PEI (polyethylenimine) condensates are honeycombed and those made with no DNA condensing agent are solid matrix type particles.²³ The increase in internal porosity obtained by the use of a DNA condensing agent was attributed to an osmotic gradient created during the W/O/W encapsulation process that promoted the flux of water into the second aqueous phase. Addition of sodium chloride to the second aqueous phase eliminates the osmotic differential and reduces the porosity and thus the DNA release rate of these microparticles.²⁵

13.6.1 POLYMER HYDROPHOBICITY

The physical and chemical properties of the polymer affect the rate of polymer degradation and DNA release; factors that increase hydropholicity of the polymer increase the DNA release rate. In the case of polyesters (e.g., PLG, PLA), hydrolysis occurs by cleavage of the ester linkages. The degradation process is bulk and homogeneous, leading to channel formation, gradual loss of mechanical structure and eventual disintegration of the matrix. In the final stage of degradation, the polymer is cleaved to fragments that are soluble in the aqueous media.⁷⁷ The accessibility of water molecules to the polymer is dependent upon its inherent hydrophobicity, a characteristic determined by the blockiness of the polymer (LLLLLGGGG, etc.), the ratio of lactide to glycolide, the type of ester linkage (L or G) and the molecular weight. For example, poly(LLLL-GGG), poly(LL)

poly(GG) are more hydrophobic and less accessible to water molecules than poly(LGGLGGGLGL). Poly-lactide has an inherent tendency to form crystalline structures and the methyl groups on the backbone of poly(LL) provide a steric hindrance to water so that PLG polymers with a higher ratio of L:G degrade more slowly. The end result of these factors is that the release rate of nucleic acid from poly(lactide) microparticles is significantly reduced in comparison to that of poly(lactide-*co*-glycolide) microparticles.^{27,46} It follows then that as the ratio of L:G increases for a PLG polymer of defined molecular weight, the degradation time increases and the release rate of DNA decreases.⁴⁶

Increased molecular weight also correlates with increased hydrophobicity. Thus, a PLG (50:50) polymer of molecular weight 12,000 Da hydrolyzes faster than a PLG (50:50) polymer of molecular weight 30,000 Da and releases DNA at a faster rate.^{4,10,46} Wang et al.,¹² confirmed this effect by demonstrating that the release rate of DNA from 50:50 PLG particles of different molecular weights was for PLG of 6,000 mwt and slowest for polymers with a mwt of 60,000 Da.

PLG particle degradation is affected by acid and base catalyzed hydrolysis of ester linkages. For example, PLG polymers with acidic, uncapped endgroups have higher inherent hydrophilicity than their corresponding end-group capped polymers.⁷⁷ Thus, nucleic acid release occurs at a faster rate from particles prepared with PLG un-capped polymers than it does from particles prepared with the corresponding capped PLG polymer.^{10,27,46}

13.6.2 CHARGE NEUTRALIZATION OF DNA

Condensation or association of nucleic acid with a cationic compound, prior to encapsulation results in a nucleic acid complex with neutralized charges and increased hydrophobicity. The chemical structure, molecular weight, and hydrophilicity of the condensing compound can determine the physico-chemical characteristics of the DNA-complex formed affect the release rate. For example, release of PEI-ODN condensates from PLG microparticles depends on the N/P ratio of the condensate; condensates with N/P ratios of 4.5 released faster than those with N/P ratios of 15 which was faster than the release obtained in the absence of condensing agent.²³

Compounds that condense DNA and increase association of the DNAcomplex with the encapsulating polymer include (dimethyldioctadecylammonium bromide (DDAB) and 1,2 diacyl-3-trimethylammonium-propane (TAP). By enhancing polymer : DNA complex interaction, the loss of nucleic acid during particle formation is minimized.⁸¹

13.6.3 INCLUSION OF HYDROPHILIC AGENTS

Factors used in the preparation of PLG particles influence DNA release if they act to increase water uptake into the particle. For example, Poly(Vinyl alcohol) (PVA), a hydrophilic polymer, is often used as an emulsifier during production of PLG particles by the W/O/W process. PVA coats or is incorporated into the polymer matrix defining the surface of the microparticle. Nucleic acid-containing microparticles prepared by a W/O/W emulsion method, using high concentrations of PVA in the aqueous continuous phase demonstrated faster DNA release rates than particles made with a reduced PVA concentration.^{8,10} In a similar fashion, incorporation of other water-soluble polymers such as PEG enhances the rate of release of nucleic acid. For example, a sustained, near-zero order rate of release ODN is observed from PEG-incorporated PLG microparticles.⁸²

13.6.4 POLYMER DEGRADATION BY DIFFUSION OR SURFACE EROSION

DNA-containing microparticles comprised of poly(anhydrides) degrade by surface-erosion and release nucleic acid as the matrix breaks apart by hydrolysis. For example, poly(FA:SA) nanoparticles demonstrate sustained DNA release for nearly 3 months, at which time nearly all of the DNA is released and the polymer has substantially degraded.³²

Particles produced by complex coacervation contain DNA embedded in a matrix that is released only in the appropriate environment. DNA/chitosan coacervates release DNA at acidic pH, a condition that promotes protonation of the amine groups; whereas, cross-linked gelatin/DNA coacervates release DNA as a function of the crosslink density and degradation of the matrix by proteolysis.⁴¹

Release of DNA adsorbed to the surface of particles is primarily dependent upon the nature of the surface-binding. For example, DNA adsorbed to the surface of PLG particles through interaction with CTAB is released at a slower rate with higher concentrations of CTAB.⁸³

13.7 CLINICAL APPLICATIONS

13.7.1 DNA VACCINES

PLG-nucleic acid particulate formulations have been widely used to promote antigen specific immune responses to DNA-encoded antigens,^{84,85} as adjuvants for protein antigens and as stimulators of the innate immune system for tumor therapy.

Activation of antigen-specific T cell responses requires that a professional antigen presenting cell (APC; e.g., dendritic cell) internalize foreign pathogens or proteins and process the proteins into small peptides that associate with human leukocyte antigen (HLA) molecules inside the APC. HLA-peptide complexes traffic to the APC cell surface and are detected by T cells. Interaction between the APC and T cell results in cytokine secretion that promotes T cell differentiation and activity. In the absence of these signals, antigen presentation can lead to T cell inactivation, tolerance, or depletion. Thus, it is advantageous to deliver DNA encoding antigens directly to professional APCs.

Particles of $0.5-1.0 \,\mu\text{m}$ are phagocytosed by immature monocytes, which differentiate into DC, and migrate to the draining lymph node.⁸⁶ Elridge et al. demonstrated that immunization of mice with PLG-encapsulated proteins results in particle uptake by APC and activation of the immune response.^{87,88} PLG particles, containing protein-encoding plasmid, are phagocytosed by DCs and macrophages in vitro.^{26,46,55} The DNA is subsequently expressed^{26, 55} and the protein processed into peptide fragments that are presented on the surface of the transfected cells in a manner that activates T cell responses.⁵⁵

Signals to activate APCs and promote T cell responses can be initiated via innate immunity. This ancient pathway is an animal's first line of defense against pathogens. Certain repeating sequences found in microbial pathogens (e.g., lipopolysaccharide, unmethylated CpG sequences on bacterial DNA) are recognized by Toll receptors found on the surface of APC.⁸⁹ In response to these signals, APCs secrete cytokines that promote recruitment and activation of inflammatory and immune cells.^{89,90} Formulations of PLG-ODN that contain unmethylated CpG sequences are highly active in stimulating cytokine secretion and innate immunity.⁹¹ This effect might ultimately be useful therapeutically to promote expression of cytokines that are cytotoxic to tumors,⁹⁰ or as adjuvants to protein vaccination. Administration of CpG and protein antigen co-encapsulated in PLG particles dramatically increased the immune response in mice over that observed following injection of encapsulated protein in the absence of CpG.⁹²

The desire to target DNA to APC provided the impetus to conduct further research in the area of immunotherapy and PLG-based plasmid delivery and the earliest published data on the use of encapsulated DNA for vaccination purposes suggested that both oral^{13–15} and parenteral⁹³ routes of administration for PLG-plasmid particles stimulates immune responses directed against DNA encoded antigens.

Oral immunization with PLG-plasmid particles stimulates mucosal immunity and the production of mucosal antibody^{13–15} that can protect mice from viral challenge.^{15,16} Oral or nasal vaccination with particles containing DNA induces antibody and T cell responses.^{58,94} Formulations containing two plasmids encoding different proteins were effective at eliciting immune responses to both antigens following oral delivery, demonstrating the versatility of this system.¹⁶

Parenteral administration of encapsulated DNA^{18,93} or DNA-coated particles^{56,57,59,95} is effective at stimulating systemic B cell and T cell responses. For example, injection of mice with encapsulated plasmid encoding HPV antigens elicits T cell responses that are specific for HPV as indicated by secretion of gamma interferon in response to HPV antigens presented on the surface of APC (Figure 13.7). Administration of encapsulated DNA encoding tumor antigens by intramuscular^{18,59} or intravenous routes,¹⁸ results in immune activation and tumor protection in mice. Intramuscular immunization of mice with DNA encoding antigens coated onto the surface of



FIGURE 13.7 Immunization of mice with PLG particles containing DNA encoding HPV antigens elicits T cell responses. Mice were immunized with HPV antigen encoding DNA encapsulated in PLG microparticles (HPV DNA) or with control encapsulated vector DNA (vector control). Splenic T cells (CD3+) were tested for their ability to respond to HPV antigens by secretion of gamma interferon in the presence of HPV antigen (HPV22) and APC by ELISPOT. Gamma interferon spot forming cells (SFC) per 10e6 CD3+ T cells are indicated on the *y*-axis. T cells are only responsive in the presence of HPV antigen, in the absence of peptide, there is no T cell repsonse.

PLG particles or with DNA encapsulated in PLG particles produces a more efficient response than that of naked DNA.^{17,57}

Activation of the immune response with DNA-particle formulations can be further enhanced by the addition of adjuvants to PLG particle formulations or by booster regimens wherein animals are primed with a first injection of particulate-DNA and then boosted with two or more injections of antigen containing formulations.

The adjuvant trehalose dimicolate (TDM) promotes the release of immune activating cytokines and the activation of macrophages.⁹⁶ Inclusion of TDM in particle formulations enhances immunity to DNA-encoded antigens encapsulated in particles over that seen with particles lacking TDM or naked DNA.⁹⁷ Adjuvants such as alum and granulocyte macrophage colony stimulating factor (GMCSF) also enhance immunity elicited by PLG-DNA.^{59,98} Prime-boost regimens that elicited immune responses by administration of PLG-DNA followed by a boost with recombinant vaccinia virus encoding the same antigen as that in the PLG-DNA formulation enhance the potency of the PLG-DNA formulation.⁹⁸

Unlike naked DNA, administration of particle-DNA has demonstrated utility in nonhuman primates. Immune responses specific for an HIV antigen were elicited by immunizing rhesus macaques with DNA-coated particles.⁹⁸ Priming rhesus macaques with DNA encoding an viral antigen encapsulated in PLG particles, followed by boosting with recombinant vaccinia virus





FIGURE 13.8 Immunization of humans with PLG particles containing DNA encoding HPV antigens elicits T cell responses. Patients were immunized with HPV encoding DNA encapsulated in PLG microparticles. Peripheral blood CD8+ T cells were tested for responses to HPV antigen by secretion of gamma interferon (ELISA) both before and after immunization (*x*-axis). Interferon gamma is indicated on the *y*-axis (pg/ml).

encoding the same antigen was effective at eliciting T cell responses that were long lasting. 99

13.7.2 CLINICAL TRIALS OF PARTICLE DNA FORMULATIONS

The value of particle based delivery for nucleic acids will be best recognized by promoting clinical benefit to patients. Thus far, the effectiveness of administering DNA encapsulated in PLG particles has been demonstrated in at least three clinical trials. In each of these studies, intramuscular (IM) injection of plasmid, encoding human papillomavirus (HPV) antigens, encapsulated in $1-2\,\mu$ m PLG particles elicited HPV specific immune responses in patients with pre-cancerous cervical or anal dysplasia (Figure 13.8).^{100,101} More recently, a placebo-controlled clinical trial demonstrated that IM administration of HPV encoding plasmid encapsulated in a similar PLG particle formulation resulted in resolution of the high-grade lesions in significantly more patients receiving the formulation vs. placebo.¹⁰² Recently it was demonstrated that immune responses can be elicited in PLG particles (Gribben et al., submitted). Future studies will include those with DNA coated PLG particles, and combinations of adjuvant and PLG-DNA particles.

13.8 CONCLUDING REMARKS

The rapid discoveries and progress that have recently been made in delivering polymer formulated DNA particulates has been spurred on by the great promise of DNA medicines and the need for protecting and targeting DNA. The most significant events in the field to date have been the advancement of PLG-plasmid DNA formulations into clinical studies. The fact that these formulations are well-tolerated and demonstrated the desired biological and clinical effects bodes well for a future regulatory approval of this novel DNA delivery system.

It is likely that these successes will act to further encourage researchers to develop even more efficacious systems for activating immune responses. Although polymer based polymer particulate formulations of oligonucleotides have not yet been tested in clinical studies, these too hold promise in the area of immune stimulation and tumor protection. Delivery of oligonucleotides that silence or activate endogenous gene expression is an area that has yet to be explored clinically with polymer particulates, but also holds great promise.

An extensive amount of data now exists to demonstrate gene expression by administration of polymer-DNA formulations *in vivo*. However, to be effective as authentic "gene therapy" based approaches wherein long term and high levels of gene expression are required, potentially for the lifetime of an individual, continued improvements are needed. It is here that addition of factors that control DNA release over extended time periods, protect DNA from nuclease attack, and aid in the nuclear uptake of increased amounts of DNA will be most valuable.

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14 Liposomal Delivery of Protein and Peptide Drugs

Vladimir P. Torchilin

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14.1 INTRODUCTION

Liposomes served as carriers of proteins and peptides in a huge variety of cases. In very general terms, one can divide the use of liposomal proteins and peptides into several big areas, each of which has its own history, achievement and failures. These areas include: (1) Incorporation of protein and peptide drugs into liposomes in order to improve their therapeutic activity in

a very broad sense of this word and to diminish various drawbacks and side effects frequently characteristics for such drugs; (2) Incorporation of various proteins and peptides into liposomes in order to modulate the immune response towards these proteins and peptides or to other antigens (for example, by protein- or peptide-modulated activation of certain components of immune systems or certain steps of the immune response development); (3) Attachment of certain proteins and peptides (usually, monoclonal antibodies or their Fab fragments) to the liposome surface in order to target liposomes (drug- or diagnostic agent-loaded liposomes) to a certain pathological areas in the body or even inside cells (using so-called transduction proteins and peptides); (4) Liposomal delivery of DNA into certain cells in order to initiate the in situ production of a therapeutically active protein (usually, enzyme) to treat a local or systemic disease; (5) Reconstitution of various membrane proteins into liposome to investigate the fine details of functioning these proteins in vivo.

Within the frame of a single chapter it is virtually impossible to even briefly consider all these variables of liposomal delivery of proteins and peptides. As a result, this chapter will completely exclude from targeted delivery of proteins and peptides as well as the use of liposomes as delivery vehicles for DNA. We will also not consider a special use of liposomes in reconstitution of membrane proteins. The role of the liposomal proteins and peptides as immunomodulators will be considered rather briefly, since there are quite a few excellent reviews already existing in this area. Mainly, this chapter will be considering the use and benefit of liposomes as carriers for protein and peptide delivery.

14.2 PEPTIDES AND PROTEINS AS DRUGS: ADVANTAGES AND ASSOCIATED PROBLEMS

Many proteins and peptides possess biological activity that makes them potent therapeutics. Enzymes represent an important and, probably, the best investigated group of protein drugs. Their clinical use has a rather long history.^{1–3} Certain diseases (usually inherited) associated with the deficiency of some lysosomal enzymes can be treated only by the administration of exogenous enzymes.^{4,5} In general, therapeutic enzymes include antitumor enzymes, which destroy certain amino acids required for tumor growth; enzymes for replacement therapy (usually digestive enzymes) for the correction of various insufficiencies of the digestive tract; enzymes for the treatment of lysosomal storage diseases; enzymes for thrombolytic therapy; antibacterial and antiviral enzymes; and hydrolytic and antiinflammatory enzymes.

Among the antitumor enzymes, the most frequently used L-asparaginase hydrolyzes asparagine via desamination of the amino acid with the formation of aspartic acid.⁶ The therapeutic action of asparaginase is based on its high requirement by some tumors, such as acute lymphoblastic leukemia. As a result, L-asparaginase became a standard tool in the treatment of all leukemia.⁷ Other enzymes of interest⁸ include glutaminase, cystein desulfatase;

cystein aminotransferase, cysteine oxidase, arginase, arginine deaminase, and arginine decarboxylase. Interesting approaches also involve the use of folatedegrading enzymes,⁹ ribonucleases and exonucleases.¹⁰ Enzymes for the therapy of lysosmal storage diseases include glucocerebrosidase, various glucosidases, phenylalanine ammonia lyase, and some others.^{4,5} Therapy with blood clotting Factor VIII can be useful for the treatment of hemophilia A.¹¹ For many storage pathologies, the treatment with purified exogenous enzymes shows very promising results. Unfortunately, therapy with native enzymes gives only a short-term relief because of their limited life in the circulation.¹

The thrombolytic therapy include the use of many different enzymes fibrinolysin (plasmin), streptokinase, urokinase (pro-urokinase), tissue plasminogen activator.^{12–14} The application of enzymes detoxifying free radical oxygen derivatives (superoxide dismutase and catalase) is recommended for different pathological conditions including the damage caused by the postischemic reperfusion.¹⁵ Many other enzymes are also being considered as promising therapeutic agents. Thus, elastase can be used in the treatment of arthritis,¹⁶ pronase is used for the treatment of spleen and liver diseases;¹⁷ collagenase and its mixture with hyaluronidase are promising in the treatment of spleen and liver diseases as well as for the treatment of postoperative scars;¹⁸ mixtures of proteases with ribonuclease and deoxyribonuclease showed good results in the prevention and treatment of postoperative pneumonia; and lysozyme is highly effective against viral diseases, including hepatitis, and in stomatology for caries treatment.^{1–3}

Antibodies against certain cancer-specific ligands can also be considered as another example of protein drugs. Two FDA-approved examples are Trastuzumab (a humanized antibody against HER-2/neu, extracellular domain of the HER-2 gene expression product characteristic of certain types of breast cancer) and Rituxan (a humanized antibody against the B-cell specific antigen CD20). Trastuzumab induces immune-mediated responses, downregulates HER-2 receptor, and promotes the production of cell-cycle inhibitors.¹⁹ Trastuzumab is well tolerated by patients, and tumor remission was observed in 60 to 70% patients who received a combined treatment with Transtuzuman and paclitaxel.²⁰ Rituxan has already proved its effectiveness against hematologic malignancies.²¹

Insulin is one of the first peptide hormones, which is commonly used for the treatment of diabetes. More recently, peptides such as somatostatin analogs (octretide, lanreotide, vapreotide) have become available in the clinic for the treatment of pituitary and gastrointestinal tumors.²² Peptide inhibitors of angiogenesis including endostatin are currently in different stages of clinical trials and show a great promise for cancer treatment.^{23,24} Research on depsipeptides has also revealed a set of potential anticancer agents.²⁵

Advances in solid-phase peptide synthesis and recombinant DNA and hybridoma technology allow for the production of unlimited quantities of clinical grade peptides and proteins. Further progress in the identification and application of new protein and peptide drugs is associated with the completion of the human genome sequence and advances in proteomics.

Still, the use of proteins and peptides as therapeutic agents is hampered by their intrinsic properties, such as complex macromolecules, which are foreign to the recipient organism. This leads to the low stability of majority of peptide and protein drugs at physiological pH and temperature. Different processes inactivate various biologically-active proteins and peptides *in vivo*. These processes include (1) non-covalent complex formation with ions or compounds that affect their native structures; (2) proteolytic degradation by endogenous proteases; (3) chemical modification by different compounds present in solution (for example, oxidation of SH-groups in sulfhydryl enzymes and Fe(II) atoms in heme-containing proteins by oxygen; thioldisulfide exchange, destruction of labile side-groups like tryptophan and metionine); (4) conformational change due to temperature, pH, high salt concentration, or detergents; (5) dissociation of protein subunits, and (6) association of protein or peptide molecules, which can convert them into inactive forms.

Following *in vivo* administration, exogenous proteins often undergo numerous changes due to external influences, as schematically shown in Figure 14.1. This leads to their rapid inactivation and elimination from the circulation mainly because of their renal filtration, enzymatic degradation, uptake by the reticuloendothelial system (RES), and accumulation in nontarget organs and tissues. At nontarget sites, a drug is wasted, in the best-case scenario. However, in many cases, the accumulation of protein and peptide drugs in healthy organs or tissues may cause severe side effects. Thus, rapid elimination and widespread distribution into nontargeted organs and tissues requires the administration of a drug in large quantities, which is often not economical and sometimes complicated due to nonspecific toxicity. A very important point is also the immune response of the macroorganism to foreign proteins containing different antigenic determinants. Specific antibodies against a given protein can rise during the life span or as a result of the repeated administration of a therapeutic protein



FIGURE 14.1 Various factors resulting in the inactivation of protein/peptide drugs in the body.

(peptide). In any case it leads to protein inactivation or even to allergic reactions, which very often make its further application impossible.

There also exist certain problems associated with the biological mechanisms of drug action. Many peptide and protein drugs as well as antibodies exert their action extracellularly, by receptor interaction. Many other, however, have their targets inside the cell. In the latter case, low permeability of cell membranes to macromolecules often represents an additional obstacle for the development of peptide- and protein-based drug formulations.

14.3 POSSIBLE SOLUTIONS: LIPOSOMES AS PHARMACEUTICAL CARRIERS

The protection of a protein against denaturating influences can be achieved via two very general approaches: (a) the steric separation of a protein/peptide and denaturating agent, and (b) the modification of a protein/peptide, which hinders its interaction with denaturating factors.² A variety of methods have been developed (known under the common name "protein immobilization") to achieve such protection via both of the mentioned approaches or their combination. Evidently, immobilized proteins are expected to possess a set of properties, which are absent in their native precursors. The most important of these properties are: (1) increased stability and prolonged activity in the body; (2) decreased immunogenicity and affinity to specific antibodies, which permits repeated administrations; (3) decreased affinity to natural inhibitors; (4) the possibility to administer the whole therapeutic dose of a protein/ peptide drug in a single injection; (5) a decrease in the total quantity of a protein/peptide drug needed for the treatment, which makes the treatment more economical. Parenterally administered immobilized/modified protein/ peptide drug should exist in the active form for a long period of time. It should also reach and penetrate an affected tissue and demonstrate improved pharmacokinetic properties and an increase in the bioavailability. In addition to the improvement of biological properties of a protein/peptide drug, it is also desirable to get a preparation, which is easier to handle.

Among many known methods to stabilize protein/peptide drugs and favorably change their bioavailability and pharmacokinetics, the encapsulation of such drugs into artificial microreservoir (microparticulate) type of carriers is frequently applied. Inside these carriers an enzyme is protected from many aggressive influences of the external medium and, in turn, does not act on different external objects. This type of systems includes liposomes, micelles, polymer microparticles, and cell hosts. The use of such carriers allows for the achievement of a higher active moiety/carrier material ratio compared to many other systems. They also provide a higher degree of protection against enzymatic degradation and other destructive factors upon parenteral administration because the carrier wall completely isolates drug molecules from the environment. An additional advantage of these carriers is that a single carrier particle is capable of delivering multiple drug species. All microparticulates have a size, which excludes the possibility of loss by renal filtration. Certain problems with microreservoir type carriers are associated with their tendency to be taken up by the RES cells primarily in liver and spleen.²⁶

One of the most popular and well-elaborated technologies to immobilize/ stabilize protein drugs and improve their pharmacological properties is their incorporation into artificial phospholipid vesicles—liposomes.

The encapsulation of proteins and peptides into liposomes has been widely studied over the years. Liposomes are artificial phospholipid vesicles, obtained by various methods from lipid dispersion in water. The problems associated with liposome preparation, their physico-chemical properties and possible biomedical application have already been discussed in several monographs.^{27–29} By now, many different methods have been suggested to prepare liposomes of different sizes, structure, and size distribution. The most frequently used methods are ultrasonication, reverse phase evaporation and detergent removal from mixed lipid-detergent micelles by dialysis or gelfiltration. To increase liposome stability towards the action of an aggressive physiological environment, cholesterol is incorporated into the liposomal membrane (sometimes up to 50% mol). The size of liposomes depends on their composition and preparation method and can vary from around 80 nm to greater than 1 um in diameter. The encapsulation efficacy for different substances is also variable depending on the liposome composition, size, charge, and preparation method. The use of the reverse phase evaporation method³⁰ permits inclusion of 50 or more percent of the substance to be encapsulated from the water phase into the liposomes. Besides, a variety of methods have been developed to obtain lyophilized liposomal preparations possessing good storage stability.³¹ The in vitro release rate of different compounds from liposomes, including proteins of moderate molecular weight, such as lysozyme or insulin, is usually under 1% per hour, under the condition that the incubation temperature sufficiently differs from the phase transition temperature of a given phospholipid. Maximal permeability of liposomes is usually observed at temperatures close to the phase transition temperature of the liposomal phospholipid. In vivo, this parameter can vary within wide limits from minutes to hours and depends on the liposome membrane composition, cholesterol content, and liposome location in the body.

From the biomedical point of view, liposomes are biocompatible, because they have no or very little antigenic, pyrogenic, allergic and toxic reactions; they easily undergo biodegradation; they protect host from any undesirable effects of the encapsulated drug, at the same time protecting an entrapped drugs from the inactivating action of the physiological medium; and, last but not least, liposomes are capable of delivering their content inside many cells. The principal mechanisms of liposome–cell interaction are presented in Figure 14.2. Different methods of liposomal content delivery into the cytoplasm have been elaborated.³² According to one of these methods, liposomes are composed of pH-sensitive components, and, after being endocytosed in the intact form, it fuses with the endovacuolar membrane under the action of lowered pH inside the endosome, releasing its content



FIGURE 14.2 Possible routes of drug-loaded liposome interaction with cells. (1) Specific adsorption; (2) Non-specific adsorption; (3) Fusion; (4) Release of an entrapped drug in the vicinity of the cell membrane with its subsequent penetration into the cell; (5) lipid exchange with the cell membrane; (6) Endocytosis that could bring the liposomal drug into lysosome (a) or allow for its release into the cytoplasm through the destabilized endosomal membrane (b).

into the cytoplasm. The method described seems to be promising for intracellular protein drug delivery, which opens unique opportunities in the enzyme therapy of diseases caused by inherited disturbances in the normal functioning of intracellular enzymes, e.g., in liver ceils.^{5,33} In addition, liposomes have been shown to fuse with microscopic pores on the cell surface (which appear as a result of natural or artificial ischemia)^{34,35} and deliver their contents including DNA into the cell cytoplasm. Liposomes modified on the surface with TAT-peptide³⁶ also deliver their cargo directly into the cytoplasm.³⁷

Liposomes loaded with protein/peptide drugs (proteoliposome) can incorporate these drugs in a variety of fashions: water-soluble proteins are entrapped into the liposomal inner aqueous space (and, in case of multilammellar liposomes, into the aqueous space between bilayers), while less soluble proteins and peptides may be incorporated into the phospholipid membrane. Intermediate cases also exist (see the scheme in Figure 14.3).

Biodistribution of liposomes is an important parameter from the clinical point of view. As with other microparticulate delivery systems, conventional liposomes suffer from rapid elimination from the systemic circulation by the cells of the RES.²⁶ Many studies have shown that within the first 15–30 min after intravenous administration of liposomes between 50 and 80% of the dose is adsorbed by the cells of the reticulo-endothelial system (RES), primarily by Kupffer cells of the liver. In order to make liposomes capable of delivering pharmaceutical agents to targets other than the RES, attempts were made to prolong their circulation lifetime. This was achieved with the



FIGURE 14.3 Protein immobilization into liposomes. Protein (P) entrapment into liposomes: (1) hydrophilic proteins can be encapsulated in the inner aqueous space of the liposome; (2) hydrophobic proteins incorporate into the phospholipid membrane; (3) proteins could be also firmly adsorbed on the liposome surface.

development of surface-modified long-circulating liposomes grafted with a flexible hydrophilic polymer (PEG being the most common example³⁸) that prevents plasma protein absorption to the liposome surface and consecutive recognition and uptake of liposomes by the RES.³⁹

A broad variety of examples show that, similar to macromolecules, liposomes are capable of accumulating in various pathological areas with affected vasculature (such as tumor, infarcts, and inflammations) via the enhanced permeability and retention (EPR) effect,^{40,41} and their longer circulations naturally enhances this way of target accumulation. Liposomal doxorubicin, incorporated into long-circulating PEGylated liposomes (Doxil[®]) demonstrates good activity in EPR-based tumor therapy and strongly diminishes the toxic side effects (cardiotoxicity) of the original drug.⁴² Evidently, long-circulating liposomes can be easily adapted for delivery of peptide (protein)-based pharmaceuticals to tumor and other "leaky" areas.

14.4 LIPOSOMAL ENZYMES

During the last two decades biologically active native compounds of protein/ peptide origin, such as enzymes, hormones, cytokines, etc., become drug of choice for the treatment of various diseases. Thus, enzyme therapy occupies an increasingly important place in the therapeutic arsenal of practical medicine. The first enzyme, lysozyme, was encapsulated into liposomes made of phosphatidyl choline, dicetyl phosphate and cholesterol more than thirty years ago, in 1970.⁴³ Later, liposomal forms of various enzymes have been prepared and investigated: glucose oxidase,⁴⁴ glucose-6-phosphate dehydrogenase, hexokinase and β -galactosidase,⁴⁵ β -glucuronidase,⁴⁶ glucocerebrosidase,⁴⁷ α -mannosidase,⁴⁸ amiloglucosidase,⁴⁹ hexoseaminidase A,⁵⁰ peroxidase,⁵¹ β -D-fructofuranosidase,⁵² neuraminidase,⁵³ superoxide dismutase and catalase,⁵⁴ asparaginase,^{55,56} cytochrome oxidase,⁵⁷ ATPase,⁵⁸ dextranase⁵⁹ as well as many other enzymes from different sources. The following parameters are usually considered as a proof of enzyme incorporation into the inner aqueous phase of liposomes or its firm and irreversible association with the liposomal membrane: the possibility of chromatographic separation of liposome-encapsulated and free enzyme; the latency of liposome-encapsulated enzymes; the correlation between protein incorporation and a change in net surface charge of the lipid bilayer.⁶⁰

14.4.1 ENZYMES FOR TREATMENT OF LYSOSOMAL STORAGE DISEASES

From the clinical point of view, the potential ability of liposome-encapsulated enzymes to enter the cytoplasm or lysosomes of live cells is of primary importance for the treatment of inherited diseases caused by the abnormal functioning of some intracellular enzymes. Despite relative rarity of these diseases, together they pose a serious medical problem.^{5,61} Lysosomal storage diseases can be divided into two main groups.⁶¹ In the first group, an abnormal (usually, deficient) enzyme is localized in the vacuolar apparatus of the cell. As a result, nondegraded substrates accumulate in the lysosomes of affected cells. Diseases, such as Tay-Sachs', Gaueher's, peroxidase insufficiency, and different mucopolysaccharidoses are typical examples. The second group includes cases when the activity of cyloplasmic enzymes is decreased; in this case, nondegraded substrates accumulate in the cell cytoplasm. Pathologies such as the Lesh–Nyhan syndrome or adenosine desaminidase insufficiency represent this group.

Unfortunately, the attempts to treat these diseases by the direct infusion of the deficient or malfunctioning enzyme were unsuccessful. Intravenous administration of purified hexoseaminidase has been tried in the treatment of Tay–Sachs' disease.⁶² The enzyme was thought to enter the affected cells of the central nervous system via endocytosis and to destroy the ganglioside GM₂ accumulated in cell lisosomes. It was found that endocytosis could not provide sufficient amounts of the enzyme inside the cells. Furthermore, predominant accumulation of hexoseaminidase was discovered in the liver instead of target CNS cells. Similar problems are linked to unsuccessful attempts with purified glucocerebrosidase and ceramidetrihexosidase therapies.^{63,64}

The use of liposome-immobilized enzymes instead of their native precursors opens new opportunities for enzyme therapy,^{65,66} especially in the treatment of diseases localized in liver cells that are natural targets for liposomes. There has been studies on the biodistribution of liposomes, which contain β -fructofuranosidase and are composed of phosphatidylcholine, phosphatidic acid, and cholesterol at 7:1:2 molar ratio.⁵² It was shown that within an hour, 50% of the administered enzyme can still be found in the circulation, but after 6 hr, 45% of the enzyme activity accumulated in the liver. The enzyme preserves its activity for a long time—25% of the administered activity can be found in the liver after 48 hr. Even after 100 hr, 5% of active enzyme is still present. Up to 50% of intracellular enzyme activity was localized in the lysosomal fraction. Similar data have been obtained for

intravenously administered liposome-encapsulated α -mannosidase⁴⁸ and neuraminidase.⁵³

 β -Glucuronidase, immobilized into charged liposomes composed of phosphatidyl choline dipalmitoyl, also demonstrated fast accumulation in the liver of experimental mice. The enzyme remained active for more than a week, associated with the lysosomes of liver cells.⁴⁶ After lysosome overloading with dextran administered intravenously into mice, the dextran content in the liver of control mice remained unchanged for 6 days, whereas rats treated with liposome-immobilized dextranase (intravenous injection) demonstrated decrease in the dextran content by 70% in 2 days.⁵⁹ Glucocerebroside β -glucosidase of human origin was encapsulated into neutral liposomes made of egg lecithin.⁶⁷ The efficiency of enzyme entrapment into liposomes is very high and the latency of the encapsulated enzyme is more than 95%. The ability of liposome-immobilized β -galaclosidase to degrade GM₁-ganglioside in lysosomes of feline fibroblasts with pathological accumulation of this substrate, has been demonstrated.⁶⁸ The native enzyme was unable to penetrate cells and to perform the therapeutic function. Later it was shown that the incorporation of liposome-encapsulated therapeutic enzymes into appropriate cells can be enhanced by rather simple methods.⁶⁹ Thus, β -glucocerebrosidase included into liposomes was predominantly captured by Kupffer cells in the liver; and the modification of liposomes with mannoside residues increased the capture because of the presence of mannose-specific receptors on target cells.

Liposomes containing β -galactosidase were successfully incorporated into the liver and spleen in the mouse model of globoid cell leukodystrophy. These liposomes were composed of lecithin, cholesterol, and sulfatide at a molar ratio of 7:2:1. Pre-injection of liposomes containing galactocebroside was followed by a single injection of the liposomes containing β -galactosidase. Since there was 70 to 80% breakdown of galactocebroside, indicating hydrolysis of galactocerebroside trapped into these liposomes by β -galactosidase.⁷⁰ These results suggest that exogenous enzyme trapped in liposomes can, therefore, be useful for the correction of accumulated compound.

The inherited abnormal metabolism of porphyrins results in porphyrias. The molecular basis of the disease can be correlated with increase in δ -aminolevulinate synthetase activity in conjunction with normal or even decreased activity of δ -aminolevulinate dehydratase. Espinola et al. (1983).⁷¹ incorporated purified δ -aminolevulinate dehydratase into liposomes. Under optimal conditions, the encapsulation efficiency could reach up to 40%, with no loss in enzyme activity.

The first clinical experiments utilizing liposome-encapsulated therapeutic enzymes were performed for the treatment of diseases of the reticuloendothelial system. Impressive results have been obtained by treatment of a patient suffering from Gaucher's disease (inherited glucocerebrosidase insufficiency, causing excessive glucocerebroside accumulation in liver lysosomes), using liposomal glucocerebrosidase.⁷² Here, therapy with the native enzyme gave no results because of its inability to penetrate cells. The administration of the enzyme encapsulated in liposomes, over a period of 13 months produced a pronounced effect, in particular a decrease in liver size with a very small amount of the active enzyme used. An attempt to treat a patient suffering from type II Pompe's disease is also encouraging. In this case, intravenous injection of 3 mg of amyloglucosidase immobilized in liposomes (170 mg of phospholipids; phosphalidylcholine, phosphatidic acid and cholesterol (in 7:1:2 molar ratio) over a period of 1 week into an affected child led to a measurable decrease in liver size.

14.4.2 ANTI-CANCER ENZYMES

Animal experiments have also clearly demonstrated the suitability of liposomes for immobilization of enzymes used for the therapy of diseases not located in the liver. L-asparaginase used for the treatment of asparaginesdependent tumors is a good example. Thus, an increase in the circulation half-life of the liposomal L-asparaginase (in lecithin-dicetylphosphate liposomes, a negative charge of the liposomes was used to inhibit the liposome interaction with cells and to keep them in the blood longer), and decrease in its antigenicity and susceptibility towards the proteolytic degradation together with the increase in the efficacy of experimental tumor therpy in mice have been shown.⁵⁶ The longevity of the liposomal L-asparaginase (in non-long-circulating liposomes) depends also on the liposome size: in large liposomes (ca. 1200 nm) the circulation time was decreased, while in small liposomes (ca. 170 nm) it was prolonged 10-fold compared to free enzyme.⁷³ The use of the liposome-encapsulated asparaginase improves the survival of animals with P1534 tumors compared to free enzyme. It is also important that the encapsulation into liposomes prevents the production of anti-asparaginase antibodies. Palmitovl-L-asparaginase was also incorporated into liposomes and demonstrated prolongation in plasma half-life by almost 10-fold, decrease in acute toxicity and improved antitumor activity in vivo.⁷⁴

14.4.3 OTHER ENZYMES

Superoxide dismutase (SOD) protects against cytotoxic effects of superoxide anions). SOD encapsulated into liposomes demonstrated an improved pharmacokinetics including longer plasma half-life and slower release of SOD, with no side effect.⁷⁵ Liposome-entrapped SOD reduces ischemia-repefusion oxidative stress in gerbil brain upon intraperitoneal bolus injection by increasing enzyme activity and decreasing membrane peroxidation in various regions of the brain.⁷⁶ Liposomes can also be used for transmembrane intracellular delivery of superoxide dismutase and catalase.^{77,78} This is extremely important for elimination of oxygen-derived free radicals inside cells, as their increased generation causes ischemia of endothelial and many other cells. Using liposomes, intracellular SOD activity can be increased

by 15-fold. Improved delivery of SOD to pulmonary epithelium using pH-sensitive liposomes⁷⁹ was demonstrated in vitro using cultured cells, and receptor-mediated endocytosis was shown to be at least partially responsible. Spray-dried powder formulations of the active SOD in DPPC liposomes mixed with disaccharides have recently been described.⁸⁰

Experimental thrombolytic therapy with the liposome-incorporated tissuetype plasminogen activator (t-PA) in rabbits with jugular vein thrombosis clearly demonstrated the benefits of the liposomal t-PA over equimolar doses of free t-PA: about 0.24 mg/kg of liposomal t-PA practically equaled the lysisactivity of a dose of free t-PA of 1.0 mg/kg.⁸¹

Many studies deal with peroxidase incorporation into liposomes and delivery of liposome immobilized enzyme into different organs and tissues.^{82,83} In particular, liposomal peroxidase was incorporated into the nerve-endings of the ray electric organ. Liposome immobilized cytochrome oxidase from bovine heart was delivered into rabbit erythrocytes.⁸⁴ Urease⁸⁵ and glutathione transferase⁸⁶ have been also obtained in liposomal form. The efficiency of intracellular cGMP formation was increased by the treatment of cells (erythocytes) with liposomes, containing the membrane form of guanylate cyclase.⁸⁷ In this case, the enzyme was delivered intracellularly using the liposome-cell fusion induced with polyethylene glycol 4000.

Cell lines (including human osteosarcoma) treated with liposome-incorporated nucleoside kinases demonstrated an increased sensitivity towards nucleoside analogues.⁸⁸

The use of liposomes for the transfer of therapeutic enzymes through the "blood–brain" barrier, which permits to deliver these enzymes into cells of the central nervous system also seems very attractive. It has been already shown that horse radish peroxidase, encapsulated into liposomes made of phosphatidylcholine, cholesterol and phosphatidic acid (7:2:1 molar ratio), acquires the ability to cross the hemato-encephalic barrier, whereas the native enzyme cannot. The presence of peroxidase in brain cells was proved by histochemical methods.⁸⁹ The same authors have shown that after injection of the liposomal glucose oxidase into the rat's tail vein, up to 5% of the enzymatic activity can be discovered in brain tissues.⁹⁰

An interesting approach to the use of liposomal enzymes is their application for antibody-directed enzyme prodrug therapy (ADEPT). ADEPT approach allows for the specific generation of active cytotoxic molecules from their inactive precursors or prodrugs in the vicinity of tumor cells. For this purpose, a conjugate of a tumor-specific antibody with an enzyme responsible for conversion of a prodrug into the active drug is targeted towards tumor, accumulates there, and convert inactive prodrug into a cytotoxic molecule right at the site of its action.^{91–93} In order to increase the efficiency of the required enzyme in the tumor, it was suggested to use not just "straight" antibody-enzyme conjugates, but rather imunoliposomes loaded with the required enzyme (immuno-enzymosomes).^{94,95} Experiments have been performed with tumor-specific liposomes bearing β -glucuronidase capable of activating anthracycline prodrugs.^{95,96}

Eventually, it was found that the encapsulation into liposomes could affect certain properties of enzymes or even cause certain undesirable side effects. Thus, the liposomal DNase I can provoke neoplastic transformation in embryonal Syrian hamster cells in culture.⁹⁷ It was also shown that the liposomal membrane can influence catalytic properties of the liposomeimmobilized β -D-galactoside $2\rightarrow 3$ sialyltransferase.⁹⁸ The probability of similar effects should always be taken into account with liposomeimmobilized enzymes (especially, membrane enzymes). Special attention has also been paid to the immunological properties of liposome-immobilized enzymes. The ability of liposomes to demonstrate in some cases adjuvant properties is already well known (see blow). Hudson et al.⁹⁹ demonstrated the enhanced immune response in mice receiving intravenous injection of liposomes containing bovine β -glucuronidase.

Liposomal protein drugs are a subject of a broad research and development. As a result, the development of production methods of liposomal proteins has become an important issue,¹⁰⁰ and various techniques of encapsulating proteins into liposomes have been described including a multiple crossflow injection technique.¹⁰¹

14.5 LIPOSOMAL PROTEINS AND PEPTIDES

14.5.1 PROTEINS OTHER THAN ENZYMES

From the practical point of view, new artificial oxygen-transporting systems capable of prolonged activity in the circulation are of special interest. Since this topic has been extensively elaborated; we will only discuss some of the proteins. For example, hemoglobin was incorporated into liposomes of different composition and this liposome formulation is called hemosomes. It was shown that the maximal quantity of hemoglobin obtained from lyzed erythrocytes incorporates into negatively charged liposomes.¹⁰² Szebeni et al.¹⁰³ have stabilized hemosomes with carboxymethylchitin. Stabilized hemosomes bind oxygen in the same way as human blood hemolysates. The acute toxicity of hemosomes was moderate in mice, the LD₅₀ was 13.8 ml hemosomes per kg weight. Polymerizable liposomes^{104,105} have also been used for hemosome preparation. Stable polymerized hemosomes are capable of the reversible binding of molecular oxygen in physiological conditions even at high flow rates.¹⁰⁴ The incorporation of allosteric effectors into hemosomes permits almost quantitative conversion of immobilized hemoglobin into the oxy-form.¹⁰⁵ Sometimes, however, liposomes can cause undesirable changes in hemodynamics including immediate hypersensitivity and cardiopulmonary distress¹⁰⁶—this was shown in pigs receiving the liposomal hemoglobin (complement activation-related pseudoallergy).

Liposomes have also been used for immobilization of some other nonenzymatic proteins, which are of interest from the clinical point of view. Kirby et al.¹⁰⁷ have described a preparation of liposomes containing blood clotting Factor VIII, which can be used for the treatment of hemophilia. Experiments have been done on the peroral administration of Factor VIII-containing liposomes into hemophilic dogs. Angiotensin II¹⁰⁸ and heparin¹⁰⁹ have also been included into liposomes.

An interesting attempt has been made to treat viral diseases with the liposome-encapsulated fragment A of diphtheria toxin.¹¹⁰ It has been shown in cell culture experiments (cells infected with the virus of subacute sclerosing panencephalitis), that the liposomes containing fragment A of diphtheria toxin decreases cell infection by 99%, whereas the free fragment A of diphtheria toxin does not influence infected cells at all.

14.5.2 PEPTIDES

14.5.2.1 Insulin

Incorporation of insulin into liposomes was done for different purposes: (i) to deliver insulin specifically to the liver, (ii) prolong insulin action in the body, and (iii) enhance the oral absorption of insulin (early studies were reviewed by Spangler¹¹¹). Later, the liposomal insulin was used for intratracheal administration.¹¹² It was shown that insulin incorporation into liposomes made of dipalmitoylphosphatidyl choline and cholesterol (7:2) resulted in an improved pulmonary uptake of insulin in rats and enhanced the hypoglycemic effect. Interestingly, similar results have been achieved after the administration of the physical mixture of free insulin and "empty" liposomes, so the exact mechanism of the enhanced activity of insulin in these experiments remained unclear.

The attempt to improve the bioavailability of the oral liposomal insulin by coating insulin-containing liposomes with chitosan for better mucoadhesion in the GI tract turned out to be successful in rats and resulted in an efficient and long-lasting lowering of glucose level.¹¹³ Similar results have been also obtained with insulin-containing liposomes coated with PEG or mucin¹¹⁴ and explained by better interaction of polymer-coated liposomes with the mucus layer and better retention of insulin under aggressive conditions of the stomach and GI (general issues associated with the preparation of the liposomal dosage form with an improved mucoadhesion for oral and pulmonary administration of peptide drugs have been recently reviewed by Takeuchi et al.¹¹⁵).

The efficiency of the oral administration of the liposomal insulin in liposomes of different phospholipid composition was also confirmed by Kisel et al.¹¹⁶ However, high variability of effects upon the oral administration of the liposomal insulin still represents a challenge. Buccal delivery of the liposomal insulin that showed encouraging results in rabbit experiments¹¹⁷ might represent an interesting alternative. The pharmacodynamics of insulin in PEG-coated liposomes upon the intravenous administration was studied in rats.¹¹⁸ PEGylated liposomes provided the strongest and the longest decrease in the glucose level supporting the hypothesis on slow release of
insulin from liposomes in the blood. The interest towards the liposomal insulin for both oral and parenteral application is still high.

14.5.2.2 Other Peptides: Cytokines

Cytokines were frequent candidates for liposomal dosage forms that have been expected to extend their lifetime in the body. Thus, the incorporation of recombinant interleukin-2 into liposomes increased its blood circulation time by 8-fold.¹¹⁹ Asialofetuin-liposomes were shown to efficiently deliver human recombinant interferon-gamma into hepatocytes in vitro.¹²⁰ Liposomal preparations of GM-CSF and TNF-alpha demonstrated improved pharmaco-kinetics and biological activity on the background of reduced toxicity in mice.¹²¹ Liposomal muramyl tripeptide was successfully used in patients with relapse osteosarcoma.¹²² Mannosylated liposomes with muramyl dipeptide significantly inhibited liver metastases in tumor-bearing mice.¹²³

The possibility of using TNF encapsulated into long-circulating PEGliposomes for the treatment of solid tumors, possibly, in combination with other cytotoxic agents was discussed in the literature.¹²⁴ PEG-coated liposomes have also been proposed for oral delivery of recombinant human epidermal growth factor.¹²⁵ Liposomal delivery of the peptide inhibitor of the transcription factor nuclear factor-kappaB was shown to significantly inhibit the proliferation of vascular smooth muscle cells.¹²⁶ Liposomal recombinant human TNF strongly suppressed parasitemia and protected against *Plasmodium berghei k173*-induced experimental cerebral malaria in mice.¹²⁷

The possibility of the topical delivery of the liposomal interferon was considered by Egbaria et al.¹²⁸ and the details of the dermal penetration of the liposomal gamma-interferon pointing the key role of the transfollicular route were investigated.¹²⁹ Topical delivery of growth hormone releasing peptide in mice was suggested,¹³⁰ such liposomes for peptide delivery may be further improved by modification with hyaluronic acid that increases their bioadhesion.¹³¹ Topical delivery of the liposomal enkephalin was demonstrated¹³² confirming the earlier finding that nonionic liposomes facilitate topical delivery of peptide drugs.¹³³

Fidler and associates^{134,135} described an interesting approach to the use of liposomal drugs in cancer therapy. They suggested the destruction of malignant cells by hyper-activated macrophages of the host. For this purpose, macrophages have been stimulated with different lymphokines. In order to increase the quantity of lymphokines delivered to macrophages, the former can be encapsulated into liposomes. In experiments on mice with inoculated tumors it was shown that liposomal lymphokines are capable of preventing metastasis formation in 70% of the animals, in comparison with 10% prevention when native lymphokines were used.

Liposomal systems have also been considered as a cytokine supplement in tumor cells vaccines since they may provide a cytokine reservoir at the antigen presentation site,^{136,137} and the benefits of the liposomal interferongamma in the generation of systemic immune responses in B16 melanoma model have been clearly demonstrated by these authors. Special attention was paid to the preservation of the normal active conformation of biologically active proteins upon their association with liposomes and subsequent dissociation. Using the recombinant human interferon gamma and negatively charged liposomes as a model, it was shown that protein association/ dissociation does not affect its native structure.^{136,137}

14.5.2.3 Miscellaneous Peptides in Liposomes

The incorporation of cyclosporin into liposomes of various compositions was shown to minimize the toxic side effects associated with traditional intravenous formulations of cyclosporin and maintain good drug activity in dogs.¹³⁸ Topical delivery of liposomal cyclosporin in murine model was also described.¹³⁹ Still, the rapid exchange of the liposomal cyclosporin between vesicles¹⁴⁰ that should result in the fast transfer of the drug into various blood components *in vivo* might negate the benefits of the liposomal form of cyclosporin.

Leupeptide (the tripeptide inhibitor of proteolytic enzymes) can be delivered into the brain by means of liposomes obtained by reverse phase evaporation from a mixture of lecithin, cholesterol, and sulfatide (4:5:1 molar ratio).¹⁴¹ The inhibitor was used for the treatment of experimental allergic encephalomyelitis in guinea pigs. The therapeutic efficiency of the liposomal inhibitor was very high as estimated by the histopathology data and the survival of experimental animals. Liposome-encapsulated inhibitors of aldose reductase (quercitine, quercitrine, AU22-284 and sorbinyl) have been successfully delivered into the ocular lens.^{142,143}

Antimicrobial and antiendotoxin cationic peptide, CM3, incorporated into liposomes was suggested for aerosol delivery, and corresponding models describing its potential distribution in lungs of patients with different breathing patterns have been developed.¹⁴⁴ Liposomes with calcitonin have been developed for intranasal delivery.¹⁴⁵ Liposomal forms of the peptide antibiotic, polymyxin B, showed certain promise in different models.^{146–148} In general, a decreased toxicity of the liposomal drugs, such as amphotericin B, was frequently noticed.¹⁴⁹

Some pH-sensitive peptides may enhance drug release from folate-targeted liposomes at endosomal pHs¹⁵⁰ thus improving drug delivery into the cell cytoplasm.

14.6 LIPOSOMES AS PROTEIN AND PEPTIDE CARRIERS FOR IMMUNOLOGICAL APPLICATIONS

The induction of strong and long lasting T-cell response, CD4+ or CD8+, is the major requirement in the development of efficient vaccines. An important aspect involves delivery of antigens to dendritic cells (DCs) as antigen presenting cells (APCs) for the induction of potent antigen-specific CD8+ T lymphocyte (CTLs) responses. Protein or peptide-based vaccines become an attractive alternative to the use of live cell vaccines to stimulate CTL responses for the treatment of viral diseases or malignancies. However, vaccination with proteins or synthetic peptides representing discrete CTL epitopes have failed in most instances due to the inability for exogenous antigens to be properly presented to T cells via major histocompatibility complex (MHC) class I molecules.¹⁵¹

Liposomes have long ago been shown to be effective immunological adjuvants for protein and peptide antigens (see a good summary of numerous studies in Refs 152 and 153). Liposomes are capable of inducing both humoral and cellular immune responses towards the liposomal antigens. Liposomes with encapsulated protein or peptide antigen are phagocytosed by macrophages and eventually end in lysosomes. There, proteins and peptides are degraded by lysosomal enzymes, and their fragments are then presented on the macrophage surface being associated with the major histocompatibility complex (MHC) class II. This results in the stimulation of specific T-helper cells, and, via the lymphokine secretion and interaction of T cells with B cells that captured free antigen. stimulation of specific B cells and subsequent secretion of antibodies.¹⁵³ In some cases, however, the fraction of the liposomal antigen can escape from endosomes into the cytoplasm (for example, when pH-sensitive liposomes are used) and in this case liberated antigen is processed and presented being associated with the MHC I complex, inducing cytotoxic T lymphocytes (CTL response). The ability to induce the CTL response provides liposomes with certain benefits when compared to traditional adjuvants (such as Freund's adjuvant) that do not induce any significant CTL response.

A variety of protein antigens have been incorporated into liposomes (such as diphtheria toxoid,¹⁵⁴ hepatitis B antigens,^{155,156} influenza virus antigens,^{157,158} tumor-associated antigens,¹⁵⁹ and many others.¹⁵² A pronounced immunoadjuvant effect of liposomes can also be seen when proteins (enzymes) or other immunogens are bound to the outer surface of liposomal membranes.¹⁶⁰

Liposomal antigens have also been used to enhance the mucosal immune response. Thus, the colonic/rectal IgA response to liposomal ferritin was significantly enhanced over the response to free antigen when cholera toxin was used as adjuvant.¹⁶¹ The protective efficiency of 30 kDa secretory protein of *Mycobacterium tuberculosis* H37Ra against tuberculosis in mice was significantly enhanced by incorporating this protein into liposomes serving as adjuvant.¹⁶² Synthetic human MUC1 peptides, which are considered as candidates for therapeutic cancer vaccines were incorporated into liposomes or attached to the surface of liposomes and in both cases elicited strong antigen-specific T-cell response.¹⁶³ Formaldehyde-inactivated ricin toxoid in liposomes was used for intra-pulmonary vaccination to create the protection against inhaled ricin with good results.¹⁶⁴ Liposomal composition incorporating Antennapedia homeodomain fused to a poorly immunogenic CTL epitope increased the immunogenicity of the construct and improved immune response (activation of CD8+ T cells), evidently because of protection of the antigen by liposomes.¹⁶⁵ Cytokine-containing liposomes have been used as vaccine adjuvants.¹⁶⁶

Oral delivery of antigens in liposomes (ovalbumin was used as a model antigen) effectively induced oral tolerance.¹⁶⁷ Liposomes with the surface-attached recombinant B subunit of cholera toxin were shown to be an effective oral antigen delivery system.¹⁶⁸ In recent developments, liposomes were successfully used for delivery of peptide vaccines and CTL epitopes to dendritic cells improving immune response.^{151,169,170} Various approaches to deliver liposomal proteins to the cytoplasm and Golgi of antigen-presenting cells were reviewed recently.¹⁷¹ In general, the future of liposomal vaccines seems to be quite promising.

14.7 CONCLUDING REMARKS

Liposomes are frequently and successfully used as carriers for protein and peptide drugs and antigens and allow for their much-enhanced pharmacological and immunological activity.

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15 Mechanisms of Cellular Drug Resistance and Strategies to Overcome it

Tamara Minko

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15.1 INTRODUCTION

The history of pharmaceutical sciences is replete with examples of attempts at increasing in drug efficacy and overcoming drug resistance. These attempts have contributed substantially to our knowledge about cellular defensive mechanisms and methods to overcome them. The present chapter aims at describing the main cellular mechanisms of drug resistance and the most well-known approaches for their suppression. This field is vast and therefore the focus will be restricted to anticancer and antimicrobial drugs.

15.2 DRUG RESISTANCE—DEFINITIONS

Before analyzing the mechanisms of drug resistance it is useful to examine the definitions of different types of resistance and explain what can be included within each definition. It should be stressed that in most cases drug resistant cells are endowed with several types of overlapping drug resistance mechanisms. Therefore it is usually difficult to draw a definite line between these mechanisms and provide an exact definition of a specific type of resistance.

15.2.1 RESISTANCE

Classically, the term "resistance" is used to describe the persistence of infection or pathology in an organism despite adequate concentrations of the drug at the site of infection. Although the mechanisms of the resistance in the whole organism and a specific cell are quite different, the term "drug resistance" can be applied to specific cells. In this case the resistance means a state of the cell when the cell fails to produce the expected response after exposure to the drug. For example, if a certain concentration of anticancer drug is applied to the tumor cell population, some cells will die, while other cells will continue their proliferation. Cells which die after the first drug exposure are called drugsensitive cells, while cells that survive the exposure are designated as resistant to the drug.

15.2.2 PRIMARY RESISTANCE

Primary drug resistance is the resistance which occurs without the organism, organ, or a separate cell ever having been in the presence of the drug. This resistance is the result of adaptation of previous generation of organism or cells to different environmental impacts that finally has been genetically fixed to form current species or cell type. This type of adaptation of whole organism is called "genotypic adaptation" and leads to the formation of current species of animals and plants based on hereditary, mutations, and natural selection. As a result of genotypic adaptation, certain cells can accumulate mutations which lead to an increased activity of certain proteins that might form a basis for the resistance to a particular or many drugs.

15.2.3 SECONDARY OR ACQUIRED RESISTANCE

Secondary or acquired resistance arises only after chronic exposure of the organism or cell to the drug. For the whole organism such acquired resistance is the particular case of a general process which is called "phenotypic adaptation"—adaptation acquired in the course of individual life. Similarly to the whole organism, an individual cell may acquire resistance to a certain drug after a single or prolonged exposure to that or similar drug.

15.2.4 INTRINSIC RESISTANCE

Intrinsic resistance exists when all members of a species are resistant to a certain drug, such as *Candida krusei* is resistant to fluconazole and *Scedosporium prolificans* to all present antifungal drugs.^{1,2}

15.2.5 CLINICAL RESISTANCE

Clinical resistance means that a drug, which was found to be very effective under in vitro experimental conditions, is not effective in the treatment of certain diseases *in vivo*. Such a situation often reflects the complexity of the whole organism which has numerous defensive mechanisms that can eliminate a drug or significantly decrease its activity even before it reaches the targeted cells. Although we will not analyze this type of drug resistance in the present chapter, this should always be taken into account when the results of an in vitro study is spread to the whole organism.

15.2.6 PUMP RESISTANCE

As we will show in this chapter, the cause of drug resistance might be related to the existence of drug efflux pumps that pump the drug out from the cytoplasm to the environment or inside special cellular organelles where it is detoxified. Despite the differences in the structure and functioning of such pumps, the net result of their action is a decrease in the drug concentration inside cells. Therefore, to provide the required concentration of a drug inside the cell, its concentration in the surrounding media is significantly increased. Such increase *in vivo* usually leads to the development of severe adverse effect in healthy organs, tissues and cells that are not a target for the treatment. We proposed to call such type of resistance as "pump resistance" to distinguish it from other types of resistance that do not require drug efflux pumps.³

15.2.7 NONPUMP RESISTANCE

In addition to pump resistance, cells can develop drug resistance that is not associated with the existence of drug efflux pumps. Certain types of drugresistant cells can survive a drug exposure even if the concentration of the drug inside the cells is maintained on a level that effectively kills sensitive cells. We named this type of resistance as a "nonpump resistance" in contrast to pump resistance that requires drug efflux pumps.^{3,4} The mechanisms involved in nonpump resistance do not interfere with drug uptake by the resistant cells. They prevent the drug effect at the level of cell organelles or processes inside the resistant cells or as in some cancer cells do not allow the drug to induce cell death. The mechanisms of such resistance will be detailed later in this chapter.

15.2.8 MULTIDRUG RESISTANCE

The term "multidrug resistance" (MDR) is usually means resistance to a broad spectrum of drugs (Fennelly, 1995). In most cases MDR develops after treatment with only a single therapeutic agent. As we will show later in this chapter, such treatment may include a prolonged exposure to a drug or even a single incident. In all cases multidrug resistant cells develop certain mechanisms of drug resistance that allow them to survive after the exposure not only to the drug that has been used for the treatment, but also to other drugs structurally related or even not related to the drug initially used. Consider a drug resistant cell which overexpresses a drug efflux pump in the plasma membrane as a result of the treatment. If, for instance, this pump can pump out water soluble molecules within a certain size range, the cell will be resistant to any kind of water soluble drug within that size range irrespective of the origin and the mechanism of action of that drug. The development of multidrug resistance is the main obstacle in the treatment of many infectious diseases and in cancer chemotherapy.

15.2.8.1 Decrease in Cytotoxicity of Anticancer Drug in Multidrug Resistant Cancer Cells

Figure 15.1 shows the results of the measurement of cellular viability after exposure of sensitive and multidrug resistant human ovarian cancer cells to an anticancer drug.⁵ The two lines of cultured cells were exposed to 12 different concentration of doxorubicin (DOX). Multidrug resistant cells were derived from the sensitive cell population by incubation with DOX. They developed a stable, genetically inherited overexpression of drug efflux pumps. These pumps decrease the drug concentration inside the cells. Therefore the dose of DOX in media required to kill 50% of cells (a 50% inhibitory concentration or IC₅₀ dose) is significantly (almost 40 times) higher in multidrug resistant cells. In addition to DOX the resistant cells also have a higher resistance to other drugs, such as taxol and camptothecin.

15.2.8.2 Development of Multidrug Resistance During Chronic Exposure to a Drug

Figure 15.2 shows the result of an intermittent adaptation experiment. Sensitive cancer cells were periodically treated with doxorubicin. The concentration of the drug was 10% of the IC_{50} dose at the beginning of the experiment and 20% thereafter. Cells were exposed to DOX for 48 h followed by 48 to 96 h recovery ("rest") periods when cells were grown in fresh media without the drug. Such schema of adaptation (intermittent adaptation) led to the development of stable multidrug resistance where the IC_{50} doses to DOX and taxol became comparable to those observed in multidrug resistant cancer cells. It is very interesting, that the difference between IC_{50} doses in sensitive and multidrug resistant cells for taxol are much higher when



FIGURE 15.1 Cytotoxicity of DOX in drug sensitive and multidrug resistant ovarian cancer cells. Means \pm SD are shown. Modified from Minko et al. (1998) *J. Controlled Rel.*, **54:** 223.



FIGURE 15.2 Development of multidrug resistance during chronic exposure to doxorubicin. Means \pm SD are shown. Modified from Minko et al. (1999) *J. Controlled Rel.*, **59:** 133.

compared with DOX (about 10,000 vs. 40 times). In this experiment cells that were tested to taxol resistance once were discarded and never used in the experiments again. It appeared therefore that such high resistance to a first treatment with taxol was developed during adaptation to another drug—DOX. If we take into account that parental sensitive cells had never experienced taxol treatment, we should classify this type of drug resistance

TABLE 15.1

Effect of doxorubicin on the growth of A2780 sensitive tumors in mice. Tumor size in square cm was measured. Means \pm SD from 3–9 independent experiments are shown. Modified from Minko et al. (2000) *Int. J. Cancer*, 86: 108

	Time, days						
Series	15	18	22	25	29	32	
Control DOX	$\begin{array}{c} 0.88 \pm 0.14 \\ 0.74 \pm 0.15 \end{array}$	$\begin{array}{c} 1.23 \pm 0.27 \\ 0.98 \pm 0.40 \end{array}$	$\begin{array}{c} 1.69 \pm 0.21 \\ 0.78 \pm 0.06^{a} \end{array}$	$\begin{array}{c} 2.35 \pm 0.44 \\ 0.84 \pm 0.11^{a} \end{array}$	$\begin{array}{c} 2.71 \pm 0.47 \\ 0.99 \pm 0.10^{a,b} \end{array}$	3.06 ± 0.48 $1.10 \pm 0.07^{a,c}$	
${}^{a}P < 0.05$ ${}^{b}P < 0.05$ ${}^{c}P < 0.00$	when compar when compar l when compa	red with contro red with treate ared with treat	ol (untreated) tu d (DOX) tumo ed (DOX) tumo	umor. r on day 22. or on day 22.			

as acquired resistance, and more precisely as multidrug resistance, stressing that MDR cells acquired resistance to many structurally different drugs. Additional experiments have shown that multidrug resistant human ovarian cancer cells used in the described experiment that acquired their resistance during the DOX exposure became resistant to another structurally non-related drug—camptothecin (Minko et al. 2002). Similar results were obtained in animal tumor model (Table 15.1). In these experiments sensitive human ovarian carcinoma cells were inoculated in the flank of athymic nude mice (these mice have a very weak immune system and therefore are often used to bear human cancer xenografts). When tumor size reached about 1 cm² (day 15 of the experiment) the mice were treated with the maximum tolerated dose of DOX. It was found that after the initial decrease on day 22, tumor size began to increase on days 29 and 32 despite continued treatment with DOX. This finding corroborates the in vitro data discussed above and indicates an increase in the tumor resistance.⁶

15.3 MECHANISMS OF MULTIDRUG RESISTANCE

Several mechanisms of multidrug resistance have been discovered. The main cellular mechanisms of drug resistance are similar in normal and cancer cells from different species and in microorganisms. They include: (i) prevention of drug entry; (ii) active efflux of drugs; (iii) the enzymatic inactivation or degradation of drugs; (iv) activation of antioxidant systems; (v) DNA repair, replication and biosynthesis; and (vi) cell death defensive mechanisms.

15.3.1 PREVENTION OF DRUG ENTRY

A microorganism can surround itself with a barrier of low permeability in order to decrease the influx of the drug into the cell. Although permeation



FIGURE 15.3 Development of a second membrane as a bacterial defense against antibiotics. Modified from Nikaido (1994) *Science*, 264: 382.

barriers alone rarely produce significant resistance, this method combined with others can produce a formidable front against drugs. This mechanism does not play a role in the multidrug resistance of cancer. The lipid bilayer structure of the cell plasma membrane presents the major permeability barrier for water soluble drugs and other substances. The permeability of this barrier is correlated with the fluidity of the membrane. Therefore to decrease the drug penetration the fluidity of the membrane could be decreased. However, bacteria cannot make this membrane much less fluid or it will start to interfere with the normal functions of the membrane proteins, so some bacteria have constructed an additional structure that surrounds the cell outside the cytoplasmic membrane (Figure 15.3). An example of this is Gramnegative bacteria, such as *Escherichia coli*, which surround themselves with a second outer membrane which functions as an effective barrier. The outer leaflet of the outer membrane bilayer is composed of an unusual lipid, lipopolysaccharide (LPS), rather than the usual glycerophospholipid found in most other biological membranes. The unsaturated fatty acid chains are what make the interior of the lipid bilayer fluid by preventing tight chain packing. All the fatty acid chains present in LPS are saturated which significantly reduces the fluidity.⁷ Bacteria with this barrier must develop a new method to bring in nutrients from its surroundings, however. For this purpose, the outer membrane contains porins, a special class of proteins, which produce non-specific aqueous diffusion channels across the membrane. The properties of the porin channels exclude antibiotics crossing them by having a very small diameter (0.7 by 1.0 nm in its most constricted portion) which slows down or completely stops antibiotic influx.

15.3.2 ACTIVE EFFLUX OF DRUGS

Active drug efflux systems can be divided into two families. The first family consists of single transmembrane protein that effluxes drugs by using

proton-motive force. The second family includes adenosine triphosphate (ATP)-binding cassette (ABC) transporters. The later mechanism is very important in multidrug resistance of cancer.

15.3.2.1 Pump Resistance

The "leak and pump" model was first proposed in 1973 by Keld Danø. According to this hypothesis, an anticancer drug diffused into the multidrug resistant cancer cell is pumped out before it can enter the nucleus. Despite opposition to the "pump" model,⁸ it continues to be the main model that explains the phenomenon of drug resistance associated with the overexpression of drug efflux pumps in plasma membrane of multidrug resistant cancer cells. Because such a type of resistance depends on the existence of drug efflux pumps that pump a drug out from the cell, we designated this type of resistance as a "pump resistant cancer cells supports the pump model.

15.3.2.2 Accumulation of Drug in Drug Sensitive and Resistant Cells

To determine what percentage of a drug accumulates in drug sensitive and insensitive cells, we incubated sensitive and multidrug resistant human ovarian carcinoma cells with doxorubicin and measured DOX concentrations in media and cell lysates. It was found that in sensitive cells about 80% of total DOX was accumulated inside the cells while about 20% of it was found in surrounding media (Figure 15.4). In contrast, in multidrug resistant cells only about 15% of applied drug were accumulated in cells, while the remaining 85% were retained in media. Further analysis showed that multidrug resistant cancer cells overexpressed the *MDR1* gene encoding ABC type drug efflux pump—P-glycoprotein (P-gp) and protein itself (Figure 15.4).

15.3.2.3 Proteins Involved in Multidrug Resistance in Human and Microorganisms

Several transporters are involved in multidrug resistance in human cells and microorganisms. Examples of such proteins responsible for multidrug resistance in human cancers and microorganisms are shown in Table 15.2 and Table 15.3. Note that most of the known proteins responsible for drug efflux are members of the ABC superfamily. The most famous and abundant proteins from this family are proteins comprising P-glycoprotein (P-gp) and multidrug resistant associated protein (MRP) families. All these proteins have common elements in their structure. The typical ABC transporter contains four or five membrane-associated domains. Two or three domains are highly hydrophobic and each consists of putative transmembrane



FIGURE 15.4 P-glycoprotein expression and intracellular drug accumulation in drug sensitive and multidrug resistant cancer cells.

segments in a-helical configuration.⁹ These transmembrane domains form the pathway for drug efflux. The other two domains are nucleotide-binding domains, which are located in the cytoplasm. They contain Walker A and B motifs and the ABC signature.

The predicted structure of the most studied P-glycoprotein is shown on Figure 15.5. P-glycoprotein consists of four domains: two transmembrane domains (TMD) are present in the phospholipid bilayer and two nucleotidebinding domains (NBD) are located at the cytoplasmic surface of the membrane and contain the ATP binding cassette (ABC). Each transmembrane domain consists of six segments—helices (1–6 and 7–12 in Figure 15.5.). The detailed amino acid sequence of P-glycoprotein is shown in Figure 15.6. Putative transmembrane segments 1-12 are underlined. All ABC transporters contain the conserved ATP binding cassette (ABC) signature and Walker A and B motifs, involved in the binding and hydrolysis of ATP are shown in bold typeface. The transmembrane helices form the transmembrane pore of P-glycoprotein—a cylindrical structure localized in the membrane. Two ATP binding cassettes are localized in cytoplasm. The protein is visualized as a cylindrical structure 100 Å wide and 80 Å deep with two globular nucleotidebinding domains, each 40 Å in diameter.¹⁰ Members of the MRP cluster contain an additional N-terminal membrane-bound domain and a structure similar to P-glycoprotein. The additional domain consists of five putative α -helical transmembrane segments.

The proposed working model of drug efflux by P-glycoprotein is shown in Figure 15.7. The residues of the transmembrane domain form the drugbinding domain (oval in Figure 15.7). The substrate (a drug) enters the lipid bilayer and interacts with the drug-binding domain. The binding of the

TABLE 15.2

Examples of proteins involved in multidrug resistance in human. Modified from: Bagetto (1997) Bull. Cancer, 84: 385, and Ramachandran and Melnick (1999) Mol. Diagnosis, 4: 81

Human gene	Protein	ABC transporter superfamily	Cell location and function
MDR1	P-glycoprotein (P-gp or gp170) (140–170 kDa)	+	Plasma membrane atp-dependent drug efflux pump
spgp	Sister of P-gp (170 kDa)	+	Expressed in bile canalicular membranes
D320	_	_	Human leukemic cem cells
MRP	MRP (190 kDa)	+	Plasma membrane and endoplasmic reticulum pump
_	P-95 (95 kDa)	_	Plasma membrane
LPR	Major vault protein (110 kDa)		Nuclear membrane transporter
VMAT	Vascular monoamine transporter (62 kDa)	_	Subcellular compartments: similar transport properties as those of pgp
Ara	Coded for by a 2.2 kb mRNA	+	Anthracycline removal protein
arx	Anthracycline associated resistance	_	Largely expressed in brain, spleen and testis
_	(7 kDa)	_	Ovarian cancer (related to cell proliferation)
h-ARSA-1	(35 kDa)	_	Expression in major organs (transport of heavy metals)
DRP	Drug resistance associated protein(50 kDa)	+	Overexpressed in drug resistant leukemia and breast cancer cell lines. Intracellular localization.
BCRP	Breast cancer resistance protein	+	Breast cancer multidrug resistant cell lines. Resistance to anthracyclines and mitoxantrone.
ATCP	ATP-binding cassette protein	+	Human placenta and different human cancers

TABLE 15.3 Examples of ABC multidrug transporters in microorganisms. Modified from van Veen and Konings (1998) *Biochim. Biophys. Acta*, 1365: 31

Cluster	Protein	Organism
P-gp cluster	pfMDR1	Plasmodium falciparum
	ehPgp	Entamoeba histolytica
	ldMDr1	Leishmania donovani
	Cdrlp	Candida albicans
	Pdr5p	Saccharomyces cerevisiae
	Snq2p	Saccharomyces cerevisiae
	LmrA	Lactococcus lactis
MRP cluster	Ycf1	Saccharomyces cerevisiae
	Yorl	Saccharomyces cerevisiae
	ltPgpA	Leishmania tarentolae
	ceMDR1	Caenorhabditis elegans
	BCECF	Lactococcus lactis



FIGURE 15.5 Predicted secondary structure of P-glycoprotein. Modified from Loo and Clarke (1999) *Cell Biol.*, 77: 11.



FIGURE 15.6 Sequence of P-glycoprotein. Putative transmembrane segments 1–12 are underlined. All ABC transporters contain the conserved ATP binding cassette (ABC) signature and Walker A and B motifs, involved in the binding and hydrolysis of ATP. Modified from Loo and Clarke (1999) *Cell Biol.*, **77:** 11.



FIGURE 15.7 Proposed working model of P-gp-mediated drug efflux. Modified from Loo and Clarke (1999) Cell. Biol., 77: 11.

substrate stimulates ATP hydrolysis. Hydrolysis of ATP at nucleotide-binding domains (NBD) leads to a conformational change in NBD that reduces the affinity for the substrate and leads to the release of the drug on the outside resulting in drug efflux.

Each drug efflux pumps has a certain level of specificity. Many drugs are transported by both P-glycoprotein and MRP efflux pumps. However, some drugs are transported only by MRP pump. P-glycoprotein, localized mostly in plasma membrane, effluxes large, hydrophobic, uncharged or slightly positively charged molecules. In contrast, MRP also transports negatively charged compounds, especially glutathione or glucuronate conjugates, and may isolate some of its substrates into intracellular vesicles.¹¹ Drugs belonging to many different chemical classes fall in the categories of substrates transported by ABC transporters. Some of these are the so-called natural product drugs, which were originally isolated from plants, bacteria, or fungi. The most commonly used natural anticancer drugs are vincristine, doxorubicin, VP-16, and paclitaxel. These drugs are very effective at the beginning of treatment. However, if treatment continues, cancer cells overexpress drug efflux pumps which drive out these drugs from the cells. This requires an increase in drug plasma concentration that in turn leads to the severe adverse side effects.

15.3.3 ENZYMATIC INACTIVATION OR DEGRADATION OF DRUGS

Inside cells drugs are subjected to the action of many intracellular enzymes. As a result, drugs could decrease their activity and/or increase their charge or solubility. The latter makes drugs available for active pumps, which could eliminate drugs from cells or transport them inside organelles for further deactivation. Intracellular drug metabolism includes two phases: phase Idegradative, where the drug undergoes oxidation, reduction or hydrolysis, and Phase II—detoxification, where the drug undergoes glucuronyl, sulfate, acetyl, glutathione, or glycine conjugation which increase hydrophilicity. In phase one biological activity of a drug usually increases, while detoxification of a drug takes place in phase two. This latter phase is the most important for the development of multidrug resistance. It includes four main types of conjugation: glucuronyl, sulfate, acetyl, and glutathione or glycine conjugations. After being taken up by the cell, conjugated drugs then may be transported outside the cell by efflux pumps or into cellular organelles for further degradation (Figure 15.8). Normally these detoxification processes are located in the liver. However, experimental data showed that cancer cells may increase the activity of certain detoxification enzymes after the exposure with anticancer drug including uridine diphosphate glucuronyl transferase and glutathione S transferase.^{6,12}

15.3.4 ACTIVATION OF ANTIOXIDANT SYSTEMS

Many anticancer drugs initiate cellular damage and cell death by the induction of free radical processes. Therefore, compensatory activation of cellular



FIGURE 15.8 Role of drug efflux pumps in drug detoxification. After being taken up by the cell, conjugated drugs then may be transported outside the cell by efflux pumps or into cellular organelles for further degradation. Glucuranyl and glutathione conjugates are often transported by the multidrug resistance associated protein.

defensive systems responsible for the limitation of such type of damage might lead to an increase in the cellular resistance. Oxygen radicals and antioxidant systems play a special role in these processes.

15.3.4.1 Free Oxygen Radicals and Lipid Peroxidation

Free oxygen radicals are generated during univalent reduction of oxygen (Figure 15.9). Under normal conditions one oxygen molecule receives four electrons and converts to water during respiration in mitochondria. Under certain conditions in the presence of iron or copper, oxygen could receive only one electron generating a superoxide radical. This radical in turn may react with hydrogen peroxide forming a hydroxyl or with hydrogen producing peroxide. These radicals are very reactive substances, which can damage proteins, nucleic acids, lipids, and other biomolecules.



FIGURE 15.9 Oxygen free radicals.

TABLE 15.4Major antioxidant systems

System (main player, enzyme)	Mechanism of action		
Cytochromes	${}^{\bullet}\mathrm{O}_{2}^{-} + \mathrm{Cyt} \cdot \mathrm{Fe}^{3+} \rightarrow \mathrm{O}_{2} + \mathrm{Cyt} \cdot \mathrm{Fe}^{2+}$		
Superoxide dismutase (SOD)	$^{\bullet}\mathrm{O}_{2^{-}}+^{\bullet}\mathrm{O}_{2^{-}}+2~\mathrm{H}^{+}\rightarrow\mathrm{H}_{2}\mathrm{O}_{2}+\mathrm{O}_{2}$		
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$		
Glutathione peroxidase	$^{\bullet}\mathrm{ROOH}+\mathrm{2G}\text{-}\mathrm{SH}\rightarrow\mathrm{ROH}+\mathrm{GS}\text{-}\mathrm{SG}+\mathrm{H_{2}O}$		
Antioxidants (α -tocopherol, steroids, thyroxine, etc.)	$\bullet O_2 + AO + O_2 + \bullet AO$		

One important process initiated by free oxygen radicals is lipid peroxidation. Lipid peroxidation damages cellular membranes destroying normal functions of intracellular organelles and finally might lead to the cell death. Lipid peroxidation is a free radical chain reaction. A hydroxyl radical attacks polyunsaturated fatty acids in biological membranes, for example linolenic acid, and forms the lipid radical. If a carbon atom in the 11th or 14th position is affected, conjugated dienes are formed. In the presence of oxygen, the lipid radical forms the lipid peroxy radical. This radical may interact with a new molecule of fatty acid RH producing lipid hydroperoxide ROOH and the next lipid radical. This triggers the chain reaction of lipid peroxidation.

Several antioxidant systems play in ensemble to protect a cell from the damage induced by oxygen free radicals. Most important are: cytochromes, superoxide dismutase, catalase, glutathione peroxidase, antioxidants (α -tocopherol, steroids, thyroxine, etc.) (Table 15.4). Superoxide can reduce oxidized cytochromes converting to slow reacting oxygen. Superoxide radicals can also be removed by the presence of specific enzyme superoxide dismutase. In this reaction two highly reactive superoxides interact with two protons giving much less reactive hydrogen peroxide and oxygen. Formed hydrogen peroxide is deactivated by glutathione peroxidase. In this reaction reduced glutathione (G-SH) is used as a reducing agent for lipid hydroperoxide (ROOH). Oxygen and lipid radicals may interact with antioxidants (α -tocopherol, steroids, thyroxine, etc.). In this reaction highly reactive lipid or oxygen radicals are substituted by low reactive radicals of antioxidants (*AO).

15.3.4.2 Activation of Antioxidant Systems After Adaptation to Anticancer Drug

The activity of lipid peroxidation on sensitive and multidrug resistant cancer cells during the treatment with an anticancer drug was studied.¹² In these experiments xenografts of human sensitive and multidrug resistant tumor were grown in the flanks of nude mice. Starting from the day 15 of the



FIGURE 15.10 Lipid peroxides in untreated tumors and tumors treated with free DOX. Modified from Minko et al. (2000) *Int. J. Cancer*, **86:** 108.

experiment the animals were treated with doxorubicin. Products of lipid peroxidation were measured in tumor tissues (Figure 15.10). It was found that initially DOX induced lipid peroxidation in both sensitive and resistant tumors (the induction of lipid peroxidation in resistant tumors was significantly less pronounced). However, after 25 days of treatment, the concentration of products of lipid peroxidation decreased as the result of the activation of antioxidant systems.

15.3.5 DNA REPAIR, REPLICATION AND BIOSYNTHESIS

Most anticancer and antimicrobial drugs directly or indirectly damage DNA. Therefore an increase in the power of cellular systems responsible for DNA repair, replication and biosynthesis leads to the increase in the cellular nonspecific resistance against many exogenous impacts including drugs and could produce multidrug resistance. Two main classes of enzymes control these processes of DNA repair and synthesis: topoisomerases and thymidine kinases. Topoisomerases are specialized in the sealing of DNA break (nicks) during DNA replication while thymidine kinases control biosynthesis of DNA. It was shown both in vitro and *in vivo* experiments that the activation of topoisomerases and thymidine kinases after the treatment with anticancer agents increased the resistance of cancer cells and tumors to chemotherapy.^{12,13}

15.3.6 Cell Death Defensive Mechanisms

15.3.6.1 Apoptosis and Necrosis

Cells can undergo two distinct types of cell death: necrosis and apoptosis. Necrosis (Gr. *Necrosis*, a killing)—accidental or pathological cell death occurs when cells are exposed to pathological conditions which may damage the plasma membrane or limit cell metabolism. These conditions are hyper-thermia, hypoxia, radiation, mechanical abrasions, and other damaged environmental impacts. Apoptosis (Gr. *Apoptosis*, falling or dropping off, Fr. *apo*, off, *ptosis*, a falling)—programmable cell death occurs under normal physiological conditions. In this case the cell is an active participant in its own death. Because of this apoptosis is sometimes called "cellular suicide." Apoptosis occurs during normal cell turnover and tissue homeostasis, embriogenesis, development of the nervous system, some immune response, etc. It is now recognized that most, if not all, normal physiological cell death occurs by apoptosis. Most anticancer drugs kill cancer cells by apoptosis.

Necrosis begins with disturbances of tissue metabolism and energy deficit. The cell cannot maintain its homeostasis. Intracellular organelles, most notably the mitochondria, and the entire cell swell. Then intracellular and plasma membranes break. The cytoplasmic contents, including lysosomal enzymes are released into the extracellular fluid. As a result *in vivo*, necrotic cell death is often associated with extensive tissue damage, edema, and inflammation.

Apoptosis begins with chromatin aggregation, nuclear, and cytoplasmic condensation. Apoptotic cells shrink and pull away from neighboring cells. This stage is similar to falling leaves that gave the name for the whole process. Then cytoplasm and nucleus divide into membrane bound vesicles which are often called "apoptoic bodies." Apoptoic bodies contain ribosomes, morphologically intact mitochondria and nuclear material. In vivo, the apoptoic bodies are phagocytized by macrophages or epithelial cells. In vitro, the apoptoic bodies swell and finally lyse. This terminal phase of in vitro apoptosis is called "secondary necrosis."

15.3.6.2 BCL2 Protein Family

The process of cell death by apoptosis is controlled by many mechanisms. Some of them (proapoptotic) initiate or promote apoptosis, others (antiapoptotic) limit the apoptotic cell death. The final fate of the cell depends on the balance between these two processes. If proapoptotic processes prevail, the cell dies by apoptosis. In contrast, antiapoptotic processes limits apoptotic cell death.

A special group of proteins—BCL2 protein family—plays a central role in apoptosis induction after drug exposure.^{14,15} The BCL2 protein family consists of two types of proteins with counter modulating functions: (1) the group that suppresses apoptosis if overexpressed; (2) the group that has the

ability to induce apoptosis.^{15,16} The expression ratio of anti-apoptotic members of BCL2 protein family to pro-apoptotic members determines survival or death following an apoptotic stimulus after chemotherapy of cancer cell lines.¹⁷ Figure 15.11 provides a schematic representation of the role of pro- and anti-apoptotic members of the BCL2 protein family in cell death. Each drug initially produces some primary disorder inside the cell, including damage to DNA, microtubules, or other cell structures. If this



FIGURE 15.11 BCL2 protein family and apoptosis.

primary damage does not lead to the cell death, the cell remains viable after the initial insult and can undergo repair and resume their proliferation. Thus, cytotoxicity of a drug depends on the translation of drug-induced damage into cell death. This translation requires the activation of specific pathways of cell death.^{12-15,18,19} The critical determinant of these pathways is the ratio of pro-apoptotic to anti-apoptotic members of BCL2 protein family (Figure 15.11). Downstream of this checkpoint are two major execution programs: (1) the mitochondrial dysfunction leading to necrosis and (2) caspase-dependent signaling pathway of apoptosis. Mitochondrial dysfunction includes a change in the mitochondrial membrane potential, production of reactive oxygen species, ATP deprivation, etc. Release of cytochrome c into the cytosol leads to the formation of apoptosome-the combination of cytochrome c, procaspase 9, dATP, and apoptotic protease activating factor-1 (APAF1). This in turn converts inactive procaspase 9 into its active form. Active caspase 9 initiates a cascade of downstream caspases that finally leads to cell death by apoptosis.

15.3.6.3 Activation of Antiapoptotic Cellular Defense in Cancer Cells During the Treatment with Anticancer Drugs

The induction of apoptosis by different anticancer agents has been studied under the wide range of concentrations of drugs and durations of drug exposure in various human cancer cells. It was found that the incubation of human cancer cells with high concentrations of anticancer drugs, or over a prolonged period of time, led to the development of drug resistance.^{3,4,13,18–20} Figure 15.12 gives an example of an increase in antiapoptotic resistance in human cancer cells incubated with doxorubicin, a traditional wellestablished anticancer drug. In these experiments the degree of apoptosis and the expression of main antiapoptotic member of BCL2 protein family (BCL2) were measured. The data show that under the low concentration of the drug, cells respond to the treatment by apoptosis. In contrast, cells that survived exposure to high drug doses overexpressed the BCL2 protein and developed resistance to apoptosis. Similar results were obtained after repeated incubation of human ovarian carcinoma cells with low doses of DOX and after treatment of mice xenografts of ovarian carcinoma cells by DOX (Figure 15.12). It was found that DOX successfully induced apoptosis in tumor tissue and decreased the tumor size up to 25 days of treatment. Starting from the 25th day, overexpression of the BCL2 protein and other antiapoptotic members of BCL2 protein family was observed in tumor tissue; apoptosis induction was attenuated and tumor started to grow progressively. Data obtained on different cell lines (sensitive and multidrug resistant ovarian, breast, prostate, leukemia, and lung cancer cells) and tumor xenografts are consistent and show that several anticancer drugs simultaneously induce cell death and activate antiapoptotic defense by overexpressing antiapoptotic members of BCL2 protein family.



FIGURE 15.12 Activation of antiapoptotic defense in cancer cells after treatment with DOX. Apoptosis induction (A, C) and expression of the BCL2 gene (B, D) in sensitive cancer cells (A, B) and tumors (C, D) treated with DOX.

15.3.7 SUMMARY

During recent decades it was found that the development of multidrug resistance in mammalian cells is a complicated multifactor process, which includes the following mechanisms that are summarized in Figure 15.13. First, the drug can be pumped out by drug efflux pumps. Second, during the transfer of drug within the cell, it can be detoxified losing its activity. Third, activation of antiapoptotic cellular defense in response to drug



FIGURE 15.13 Main mechanisms of multidrug resistance in cancer therapy.

exposure significantly limits cell death induction. Fourth, in many cases disturbances of cellular metabolism induced by a cytotoxic drug initiate free radical damage, which however may not lead to cell death induction as a result of the activation of antioxidant systems. Fifth, an activation of DNA repair and synthesis substantially limits the damage introduced by the drug. The synergistic action of the mentioned ensemble of defensive mechanisms finally leads to the development of drug resistance and limit the therapeutic efficacy of the drug. Considering the complexity of cellular defensive mechanisms it is understandable how in many cases our bodies can survive high drug exposures and it seems amazing that certain anticancer drugs can kill cancer cells at least at the beginning of chemotherapy. It is also logical to hypothesize that suppression of cellular drug defense will significantly increase the efficacy of cancer therapy and possible antimicrobial treatment. Several promising directions will be discussed further in this chapter.

15.4 MODULATION OF DRUG RESISTANCE PHENOTYPE

Although several methods have been proposed during the past decades to modulate drug resistance, none of them has found wide application in clinics. The reason for such failure is that in most cases the method is based on the suppression of a single cellular defensive mechanism while leaving others to function. Generally speaking the suppression of only pump or nonpump cellular resistance will not result in a significant increase in cell death. For example, if we suppress pump resistance and increase on this basis drug concentration inside the cell, this increase will stimulate the activation of cellular nonpump resistance which in turn will significantly limit or even prevent cell death. Consequently, the net result of our efforts to suppress pump resistance alone will be substantially limited by the adaptive activation of nonpump resistance. Therefore, to effectively suppress drug cellular resistance both pump and nonpump resistance should be simultaneously blocked. In the second part of the chapter we will examine existing methods for such suppression and evaluate new directions in this matter.

15.4.1 METHODS OF MODULATION OF MULTIDRUG RESISTANCE PHENOTYPE

Known methods of modulating drug resistance are summarized in Table 15.5 Most of them are attempts at modulating pump resistance. The simplest way to increase the drug concentration inside cancer cells is to increase the plasma level of the drug. However, high concentrations of most drugs induce severe adverse side effects on healthy organs, tissues, and cells. The problem of adverse side effects is especially important in case of cancer chemotherapy by highly cytotoxic drugs. To overcome the undesired toxicity to healthy organs several methods have been proposed including drug targeting, bone marrow transplantation and introduction of the MDR1 gene into bone marrow cells.

15.4.1.1 High Dose Chemotherapy

Targeting a drug specifically to cancer cells and/or specific organelles inside the cells permits the internalization of substances preferably by targeted cells and drug release in targeted organelles (e.g., lysosomes, mitochondria, nucleus, etc.). As a result the targeted drug acts like a "magic bullet" selectively killing the cancer cells. In addition, at low drug plasma concentrations, selective rapid uptake of drug by targeted cells or organ decreases the overall plasma/tissue ratio and minimizes adverse side effect to nontargeted organs. Current approaches to molecular targeting to different cells, organs and tissues are described in reviews.²¹ However, targeting alone cannot completely suppress cellular drug resistance because in most cases it does not affect nonpump resistance.

Bone marrow transplantation is used to increase the resistance of the whole organism to high doses of cytotoxic drugs or radiation during cancer therapy.^{22,23} This method is aimed at substituting damaged bone marrow in order to compensate the disturbances in the blood elements composition that often limits cancer therapy. Another method of prevention of bone marrow damage is based on the introduction of the MDR1 gene into the bone marrow cells.^{24,25} The *MDR1* gene encodes the P-glycoprotein drug efflux pump and protects the cells from high doses of toxic drugs. This original method utilizes naturally occurring pump resistance to protect the most damaging cells and therefore increase the drug dose during cancer chemotherapy. Although these methods can potentially increase the survival of the whole organism during extreme cancer treatments, they cannot be considered as true methods of modulating pump resistance because they do not suppress pump resistance directly. They just increase the survival of critical, and usually most sensitive to adverse effects of the treatment, normal cells in the hope that the whole organism will survive the procedure while cancer cells will die. A significant limitation for the practical use of such methods is a progressive increase in drug resistance of targeted cells in parallel with the increase in the drug concentration that requires progressive increase in the drug dose which in turn imposes adverse side effects.

15.4.1.2 Biochemical Modulation

Biochemical modulation of pump drug resistance is aimed at direct suppression of drug efflux pumps. Several approaches have been tested recently (Table 15.5), including coadministration of calcium channel blockers, quinolines, plant alkaloids, steroids, antiestrogens, surfactants, cyclosporins, etc. with the main cytotoxic drug.^{26–28} The idea behind such combined treatment is to suppress pump resistance by inhibiting the normal functioning of the drug efflux pumps in multidrug resistant cells. This is achieved mainly by limiting their energy supply, conformation changes or changes in physicochemical properties of surrounding plasma membrane that will interfere with drug efflux. Many products capable of reversing MDR produce severe toxic effects
TABLE 15.5Methods of modulating the multidrug resistance phenotype

- High dose chemotherapy
 - Drug targeting
 - Bone marrow transplantation
 - Introduction of MDR1 gene into bone marrow cells
- Biochemical modulation
 - Calcium channel blockers (verapamil, tiapamil, nifedipine, prenylamine)
 - Quinolines (chloroquine, quinine, quinidine)
 - Plant alkaloids (vindoline)
 - Steroids (progesterone)
 - Antiestrogens (tamoxifen)
 - Surfactants (Tween-80, cremophor-EL)
 - Cyclosporins (cyclosporin A, PSC-833)
- Gene therapy
- Antisense oligonucleotides
- New drug delivery systems

such as blocking of ion channels or modulation of other normal physiological functions. The cyclosporins initially showed promise as potent, less toxic agents that inhibit MDR. However, the immunosuppressive activity and nephrotoxicity of cyclosporins limit the usage of this group of drugs.

15.4.1.3 Gene Therapy

Gene therapy of MDR now is mainly based on the transfection of cancer cells by antimessenger oligonucleotides targeted to the binding sites of mRNA encoding the main drug efflux pumps. When technical and delivery problems are resolved, this method may be an interesting way to clinically reverse MDR. A new approach based on the use of antisense oligonucleotides (ASO) is now under test for suppressing pump and nonpump resistance.^{3,29,30} Small, chemically modified strands of DNA, the so-called "oligonucleotides," are engineered in a sequence that is exactly opposite (hence, "anti") to the coding ("sense") sequence of mRNA and are referred to as "antisense oligonucleotides or ASO." Blocking or decreasing protein synthesis by ASO includes several steps. The main ones are: (1) ASO bind to the corresponding segment of mRNA forming a duplex and preventing the reading of information and the subsequent synthesis of specific protein, (2) enzymatic degradation of the mRNA portion of the duplex, releases the ASO for further binding to other mRNA molecule, (3) after a certain period of time, ASO are exocytically ejected by the cell into the blood stream and excreted by the kidney. Advantages of ASO therapy include high specificity, selective knockout of a single critical target, potential for increased efficacy and reduced side effects.

However, many problems are encountered when oligonucleotides are used in cellular systems and *in vivo*. Two major problems associated with ASO might be solved by using of drug delivery systems and chemical modification of oligonucleotides: (1) rapid degradation in biological fluids and in cells by exoor endonucleases and (2) poor penetration by diffusion across the cell membrane. Different strategies have been proposed to reduce the degradation and/or to increase the intracellular penetration and the cytoplasmic release. Chemically modified oligonucleotides, oligonucleotides associated to virus and the use of synthetic carriers such as cationic liposomes or nanoparticles can increase cellular penetration and resistance to nuclease. These strategies can be used alone or in combination. ASO of second generation have been designed to achieve three main goals: (1) high nuclease resistance, (2) the ability to cross the cellular membrane and (3) high binding affinity and specificity for the target sequence. Nuclease resistance is usually achieved by the modification of the normal phosphodiester backbone (e.g., phosphorothioates, methyl phosphonates), incorporation of 2'-O-methyl (OMe) nucleosides (2'-OMe-RNA) or different terminal modifications (e.g., 3'-aminopropyl modification). Improved transport through the cellular membrane can be achieved by the use of a carrier molecule linked to the antisense oligonucleotide (e.g., cholesterol), the use of transfection reagents (cytofectine, DAC 30, poly-imine, etc.) or backbone modification to more lipophilic linkages (methylphosphonate). However, the most radical increase in the ability to penetrate cellular membrane can be achieved by the use of drug delivery systems. Such a drug delivery system should provide delivery of ASO to two main intracellular targets: nuclei and mitochondria.³¹

Macromolecular drug delivery systems designed to overcome or suppress multidrug resistance include systems based on micelles, microspheres, nanoparticles, liposomes and biodegradable polymers.^{32,33} Modulation of drug resistance, using "passive" and/or "active" approaches can be achieved via the macromolecular delivery systems.

15.4.2 PASSIVE APPROACHES TO MODULATION OF DRUG RESISTANCE

"Passive" approaches use two main processes: (1) the so-called enhanced permeability and retention (EPR) effect and (2) internalization of macro-molecules by endocytosis.

15.4.2.1 Enhanced Permeability and Retention (EPR) Effect

The EPR effect is the result of the increased permeability of the capillaries of the tumor vascular system to circulating macromolecules combined with limited lymphatic drainage from the tumor interstitium.^{34–36} Low molecular weight drugs coupled with high molecular weight carriers are inefficiently removed by lymphatic drainage and therefore accumulate in the tumor. Therefore, EPR effect provides a passive tumor targeting allowing the increase of drug concentration in tumors and preventing adverse side effects on healthy

organs. A significant drawback of using the EPR effect as a passive tumor targeting approach is that it works only in so-called "solid" tumors, where cancer cells are localized in one limited space. However, such types of tumor can be effectively removed by cytoreductive surgery. It is the therapy of spreading tumors and metastases that present the challenge for the chemotherapy. Unfortunately, the passive targeting approach will not work for these types of tumor because the EPR effect does not develop for spreading tumors. Therefore, therapy of such tumors requires active targeting. Theoretically any high molecular weight water-soluble drug carrier including water-soluble polymers, liposomes should exhibit "passive targeting."

15.4.2.2 Accumulation of Polymer-Bound Drugs in Solid Tumor

Two polymer-bound drugs have been used to treat mice xenografts of human ovarian tumor. The first polymer was designed in the laboratory of Dr. Kopecek and included anticancer drug doxorubicin bound to N-(2-hydroxypropyl)methacrylamide (HPMA) polymer with molecular mass about 20 kDa.^{5,6,12,13,18,32,33,37} The second polymer included anticancer drug camptothecin bound to polyethylene glycol polymer with molecular weight about 5 kDa.^{20,38} The organ distribution of both polymers was studied in a similar model of mice bearing xenografts of human ovarian carcinoma. The distribution of HPMA copolymer-bound DOX is shown in Figure 15.14. The results show that both polymers provide passive drug targeting to the solid tumor. However, the efficacy of this type of targeting increases with the increase in molecular weight of polymeric carrier.



FIGURE 15.14 Organ distribution of HPMA copolymer-bound DOX. Modified from Minko et al. (2000) Int. J. Cancer, 86: 108.

15.4.2.3 Internalization of Macromolecules by Endocytosis

The second mechanism that provides a passive way to overcome existing multidrug resistance is based on the phenomenon of different internalization of low and high molecular weight drugs. Low molecular weight drugs enter cells by simple diffusion and initially accumulate near the plasma membrane where they are actively pumped out by active drug efflux pumps. In contrast, high molecular weight polymeric therapeutics are internalizing by endocytosis in membrane-limited organelles (endosomes) and released after the fusion of endosomes with lysosomes and hydrolysis of a bond between the drug and polymeric carrier. Therefore endosomes protect the drug from drug efflux pumps and detoxification inside the cell in the initial stage of its journey. However, because endocytosis is a relatively slow process and requires significantly higher concentration of drug–polymer complex outside the cell, cytotoxicity or specific efficacy of such macromolecular composition is significantly lower when compared with low molecular drugs.⁵

15.4.3 Active Targeting Approaches to Modulation of Drug Resistance

Active targeting approach provides at least two main advantages over the passive approach. First, it targets cancer cells whether in a specific organs or metastasizing tumor within the body. Second, it significantly facilitates endocytosis by interacting as a ligand with existing extracellular receptor and therefore significantly improving drug specific activity or cytotoxicity.

15.4.3.1 Molecular Targets for Modulation of Multidrug Resistance

Several molecular targets are now used to target specific organs, tissue and cells including antibody and their fragments targeted to specific cells, biological pairs like avidin–biotin, carbohydrates–lectins, ligands to specific receptors overexpressing on plasma membrane of targeted cells, etc.^{21,39} In the present chapter we will focus specifically on molecular targets that allows modulation of cellular mechanisms of drug resistance, pump and nonpump.

15.4.3.2 Modulation of Pump Resistance

Because the main mechanism of pump resistance is the overexpression of drug efflux pumps, it is natural to target specific types of these pumps overexpressed in a particular cell type or microorganisms. Two main families of ABC transporters—P-glycoproteins and multidrug resistance associated proteins are the most commonly used targets to suppress pump resistance. We have already mentioned above small molecules that have been co-administered with the drug in order to suppress multidrug resistance. The more comprehensive way is the combination of a drug, carrier and a suppressor of pump resistance in one drug delivery system targeted to specific cells. Antisense oligonucleotides against P-glycoprotein and MRP protein

mRNA.^{3,29,30} and small interfering RNA (siRNA)^{40,41} have been used to suppress pump resistance. The following example illustrates how the suppression of MDR1 mRNA overexpression by liposomal ASO increases the toxicity of anticancer drug.

15.4.3.3 Modulation of Pump Resistance by Liposomal Antisense Oligonucleotides

We developed a liposomal drug delivery system which consisted of liposomes as carriers, doxorubicin as an anticancer agent and ASO targeted to MDR1 mRNA encoding humans P-glycoprotein.³ Measurement of cytotoxicity and expression of P-glycoprotein showed that suppression of this protein resulting in 20-fold decrease in the IC₅₀ dose indicating a significant increase in the cytotoxicity of the drug (Figure 15.15A).

15.4.3.4 Modulation of Nonpump Resistance

Several methods were developed to suppress cellular nonpump resistance. In most methods the focus was on the suppression of cellular antiapoptotic defense. Two approaches demonstrated the highest efficacy: (1) ASO targeted to antiapoptotic members of BCL2 protein family and (2) fragments of proapoptotic members of this family to suppress the activity of antiapoptotic members. The BCL2 protein family is characterized by specific regions of homology termed BCL2 homology (BH1, BH2, BH3, BH4) domains. These domains are critical to the functions of these proteins, including their impact on cell survival and their ability to interact with other family members and regulatory proteins. It was found that the BH3 domain of proapoptotic proteins from the BCL2 family is responsible for the induction of apoptosis. Furthermore, expression of small-truncated derivatives of BAK protein containing the BH3 domain was sufficient for its cell killing activity.



FIGURE 15.15 Suppression of cellular drug resistance to doxorubicin (A) and camptothecin (B) by liposomal ASO targeted to P-glycoprotein mRNA (A) and by polymer-bound synthetic BH3 peptide (B). 1, free drug; 2, liposomal (A) or polymer bound (B) drug combined with ASO (A) or BH3 peptide (B).

Moreover, it was found that short synthetic peptides, corresponding to the minimal sequence of BH3 domain when bound to the antiapoptotic BCL-2 family proteins, suppress the cellular antiapoptotic defense.^{4,19,42–44} Therefore, BH3 peptide can potentially improve traditional therapy of ovarian cancer by decreasing the resistance of cancer cells to chemotherapeutic agents.

15.4.3.5 Modulation of Nonpump Resistance by Synthetic BCL2 Homology 3 Domain (BH3) Peptide

To test the hypothesis that BH3 peptide can be used to suppress nonpump resistance, we developed a polymeric drug delivery system containing PEG polymer as a carrier, camptothecin as an anticancer drug and BH3 peptide as a suppressor of antiapoptotic cellular defense.²⁰ Application of free camptothecin induced the overexpression of BCL2 protein that limited apoptosis induction and cytotoxicity of the drug. In contrast, the application of the constructed drug delivery system prevents of the overexpression of BCL2 proteins and led to a great increase in toxicity—the IC₅₀ dose decreasing 100,000 fold (Figure 15.15B).

15.4.3.6 Novel Proapoptotic Drug Delivery Systems

Separate suppression of pump and nonpump cellular resistance led us to construct a novel targeted proapoptotic anticancer drug delivery system which includes five main components: (1) targeting moiety, that directs whole drug delivery system exclusively to cancer cells; (2) a carrier, that binds the components together and facilitates the solubility of the whole complex; (3) an anticancer drug as an apoptosis inducing agent; (4) suppressor of pump resistance; and (5) suppressor of nonpump resistance.^{3,20} The investigation of such drug delivery system and its components showed that simultaneous apoptosis induction and suppression of pump and nonpump resistance increased the cytotoxicity of the anticancer drug to the extent that could not be achieved by individual components of the system used separately. Moreover, the targeting moiety allows directing such exceptionally toxic agents specifically to cancer cells preventing serious adverse side effects. For details the reader is referred to corresponding experimental papers and reviews.^{3,20,39}

15.5 CONCLUSIONS

In current pharmaceutical practice great reliance is placed upon the use of small molecular weight therapeutics. The relative simplicity of such therapeutics and their high specific efficacy supports the interest of major pharmaceutical companies toward such therapeutics. However, simple drugs usually demonstrate a success at the initial stage of therapy of cancer and microbial infections. Microorganisms and cancer cells rapidly develop resistance to therapy which generally requires the use of an additional drug and/or

increase in drug concentration often leading to severe adverse effects. We strongly believe that the enhancement of treatment can only be achieved by using cocktail drugs that combine in one drug delivery system the anticancer drug, a targeting moiety and suppressors of pump and nonpump cellular resistance.

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16 Transporters as Molecular Targets for Drug Delivery and Disposition

Ikumi Tamai

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References

16.1 INTRODUCTION

Oral administration of drugs is desirable for the treatment of chronic diseases. However, many drug candidates fail to achieve their therapeutic potential due to poor bioavailability caused by low membrane permeability, low solubility, low chemical and biological stability in intestinal tissues, and/or high hepatic extraction after absorbtion to portal vein¹ (Figure 16.1). Among these factors, solubility could be overcome by improved formulation by utilization of solubilizing excipients such as cyclodextrins and others.² Biological drug stability in the intestinal lumen can be also improved by using inhibitors for the metabolic enzymes. The choice of protease inhibitors will depend on the structure of drug molecules.³ However, membrane permeability across the intestinal epithelial cells is hard to improve without modification of the chemical structure of the compound of interest. In such a case, a prodrug strategy that will increase the lipophilicity of an active compound was applied to increase diffusional membrane permeability.⁴ However, prodrug strategy might not be adequate for drug development, due to the requirement of repeated preclinical and clinical studies, since the prodrug will be a new chemical entity.

In many cases, specific strategies are often required for orally active drugs whose membrane permeabilities are not high enough to exhibit sufficient oral bioavailability. In this chapter a novel experimental approach to improve



FIGURE 16.1 Factors affect intestinal absorption of drugs. After administration by oral product, drug molecules are needed to be disintegrated, move from the stomach to small intestine, be dissolved, and permeate the intestinal epithelial barriers for the absorption. So, solubility, membrane permeability, and sometimes stability will be important factor to determine intestinal absorption.

the membrane permeability of the intestinal epithelial cells by using biomaterials that can activate permeability based on the membrane transport mechanism are discussed.

16.2 STRUCTURE AND FUNCTION OF INTESTINAL EPITHELIAL BARRIERS

16.2.1 PATHWAYS TO CROSS INTESTINAL EPITHELIAL BARRIERS

First of all, the mechanisms of the transport across the intestinal epithelial barriers have to be understood. Figure 16.2 shows the pathways of the transport across the barrier of the intestinal epithelial cell monolayers. First, the pathways are divided into two types, namely transcellular pathway and paracellular pathway. In the case of a paracellular pathway, drug molecules cross the barrier by moving the space between the epithelial cells. Although the adjacent epithelial cells are usually attached by tight junction to each other, they are thought to allow small compounds to pass through the barriers. On the other hand, a transcellular pathway means that drug molecules cross the epithelial cell membranes that face the intestinal lumen (apical membrane) into the cells, and then out of the cells into the blood stream across



FIGURE 16.2 Structure and pathways of the intestinal epithelial barriers. To overcome the intestinal epithelial barrier, drug molecule can cross the barrier via the paracellular and/or transcellular pathways. Transcellular pathways include simple diffusion and carrier-mediated transport mechanisms.

the basolateral membranes. So, in the case of a transcellular pathway, drug molecules need to cross both the apical and basolateral membranes to be absorbed. Here, usually apical membrane permeation is more important as the rate-limiting step. Accordingly, to increase the absorption of drugs across the intestinal epithelial barrier, we need to improve the permeability via the paracellular pathway or transcellular pathway, especially through the apical membranes.

16.2.2 PARACELLULAR PATHWAYS

It is possible to enhance paracellular pathways using absorption enhancers.^{5,6} However, the strategy of using an absorption enhancer is often related to toxicity, because these agents forcibly open the paracellular space. Various compounds in the intestinal lumen that are not preferred to be absorbed as well as the targeted drug compound will cross the barrier nonspecifically.

16.2.3 TRANSCELLULAR PATHWAYS

The other way to improve intestinal absorption is to enhance the transcellular permeability. The mechanisms for transcellular permeation are divided to two mechanisms, one is simple diffusion and the other is carrier-mediated transport. The carrier-mediated transport mechanism is usually functional for hydrophilic compounds such as hexoses, amino acids, water-soluble vitamins present in the intestinal lumen taken as foods, and the specific transporter proteins are required for the selective absorption across the membranes. On the other hand, simple diffusion is not mediated by such transporters and cross the cell membrane by dissolving into the phospholipid bilayer to the opposite side. Accordingly, simple diffusion is not as specific as carriermediated transport and is affected by the lipophilicity, molecular size, and hydrogen bonding of the crossing molecules. For organic weak acids and organic weak bases, un-ionized forms of compounds cross the membrane by simple diffusion according to the pH-partition hypothesis, since ionized forms of compounds are too hydrophilic to cross the lipoidal cell membranes. Based on pH-partition hypothesis, acidic compounds apparently exhibit higher permeability at lower pH, while cationic compounds are less permeable at lower pH. Since it is also true that many drug molecules are ionic compounds, pH should significantly affect the membrane permeability of drugs by simple diffusion.

16.2.4 CARRIER-MEDIATED TRANSPORT IN INTESTINAL EPITHELIAL CELLS

16.2.4.1 SLC Transporters

Carrier-mediated transport is due to membrane transporter proteins. In the small intestine, various transporter proteins are expressed for efficient intake of nutrients and for excretion of xenobiotics or unnecessary metabolic

products from intestinal epithelial cells into intestinal lumen. The digestive products of carbohydrates, hexoses, are absorbed by sodium-dependent hexose transporter SGLT1 and by sodium-independent GLUT5 and GLUT2 transporters.⁷ Proteins are digested to amino acids, dipeptides, and/or tripeptides in the gastrointestinal tracts. Amino acid transporters such as LAT2, y+LAT1, Asc-1 and others are expressed in the intestinal epithelial cells for those digested amino acids. In the case of dipeptides and tripeptides, they are absorbed by peptide transporter PEPT1. Interestingly, there are various amino acids transporters selective for each amino acid, while di- and tri-peptides are absorbed commonly by a single transporter PEPT1. Since the number of amino acids that constitute proteins are 20, the theoretical number of di- and tripeptides would be estimated to be 400 and 8000, respectively. Accordingly, PEPT1 is considered to exhibit broad substrate selectivity. Interestingly, substrate selectivity of PEPT1 is strict in the point of size of the peptides, since it does not accept larger peptides (tetrapeptides or larger ones) or amino acids. Bile acids are absorbed by IBAT/ASBT (ileal bile acid transporter/apical sodium-dependent bile acid transporter) in a sodium-dependent manner. Monocarboxylic acids such as lactic acid and pyruvic acid are absorbed by monocarboxylate transporter MCT1 in a pHdependent manner. These are just examples of transporters expressed in the small intestine. They are essential for absorption of nutrients and are now classified as solute carrier (SLC) transporters.⁸ If you need more information on these transporters, the details of the classification and known molecules of transporters are available from the website http://www.gene.ucl.ac.uk/ nomenclature/. Here, an important point is that certain drugs are substrates of these transporters and indeed the intestinal absorption of drugs are due to the specific transporters in small intestine.⁹ So, by utilizing these transporters, permeability of drugs across the intestinal epithelial cell membranes can be improved.

16.2.4.2 ABC Transporters

Other type of membrane transporters are characterized by excluding the substrates out of the cells back into the intestinal lumen, thereby decreasing the intestinal absorption of drugs. P-Glycoprotein was first found as the cause of the multidrug resistance of cancer cells to chemotherapeutic drugs, MRP2 (multidrug resistance associated protein), and BCRP (breast cancer resistance protein) are examples of those efflux transporters. These transporters utilize hydrolysis of adenosine triphosphate (ATP) as the driving force to exhibit transport activity and commonly have ATP binding cassette (ABC) motif in their structures.^{1,10} So, they are called ABC transporters, in comparison with SLC transporters as described above. ABC transporters such as MDR1, MRP2, and BCRP accept various compounds as substrates by having broad substrate selectivity and are expressed at the apical membranes of intestinal epithelial cells. Accordingly, the inhibitors of ABC transporters can improve absorption of their substrates. More information on ABC transporters is also available from the web site as described above for SLC transporters.

By understanding the mechanisms of the membrane transport of drug molecules across the intestinal epithelial cell membranes, several strategies to improve membrane permeability could be possible. In this chapter, one example of the method to improve membrane permeability by activating the PEPT1 transporter by biomaterial is described.

16.3 CHARACTERISTICS AND INTESTINAL ABSORPTION BY PEPTIDE TRANSPORTER PEPT1

16.3.1 PROTON-OLIGOPEPTIDE TRANSPORTER (POT) FAMILY

PEPT1 (SLC15A1) is a member of a family of proton-dependent oligopeptide transporters (POT family) and is classified to the SLC15A family by human genome nomenclature organization (HUGO).¹¹ There are four members in the SLC15A family, which are PEPT1 (SLC15A1), PEPT2 (SLC15A2), PHT2 (PTR3, SLC15A3), and PHT1 (hPTR4, SLC15A4). SLC15A family members accept di- and tripeptides and do not accept tetrapeptides or larger peptides as substrates. Histidine peptide transporters PHT1 and PHT2 transport amino acids such as histidine as well as di- and tripeptides, whereas PEPT1 and PEPT2 do not transport any amino acids. Structurally, they have twelve transmembrane domains with N- and C-terminals facing the cytosol, which is common to many membrane transporters. PEPT1 in humans is composed of 708 amino acid residues and is expressed in the apical membrane of intestinal epithelial cells (Figure 16.3). PEPT1 and PEPT2 utilize a proton gradient as the driving force for their active transport, and pH is an important factor in evaluating their transport function. Although both PHT1 and PHT2 are also thought to be activated by proton gradient, precise mechanisms remain to be clarified. Among these transporters, PEPT1 is expressed strongly in small intestine and is thought to be important for the absorption of di- and tripeptides. On the other hand, PEPT2 is important for the reabsorption of these peptides across the renal proximal epithelial cells. For PHT1 and PHT2, limited information is available in the roles of intestinal absorption for peptides and/or other compounds.¹² So, now most intestinal peptide transport studies are focused on PEPT1 and in this chapter the strategy of delivery of peptide-mimetics is also focused on PEPT1.

16.3.2 SUBSTRATE SELECTIVITY AND PH-DEPENDENCE OF PEPT1

The interesting point of PEPT1 is its broad substrate selectivity. It accepts not only di- and tripeptides as natural substrates,¹³ but also peptide-mimetic drugs, such as β -lactam antibiotics, angiotensin converting enzyme inhibitors,¹⁴ the antiviral drug valacyclovir,¹⁵ and the anticancer drug bestatin



FIGURE 16.3 Structure and intestinal apical membrane expression of PEPT1. PEPT1 has twelve trans-membrane domains and is expressed at the apical membrane of intestinal epithelial cells.



FIGURE 16.4 Substrates of peptide transporter PEPT1. Dipeptide and tripeptides in the intestinal lumen as the digestive products of protein are natural substrates of PEPT1. Synthetic peptide-mimetics such as some of β -lactam antibiotics are also substrates of PEPT1.

(Figure 16.4).9 Basic structure of the substrates of PEPT1 should include one or two peptide bonds and amino- and carboxyl-terminals. Peptide-like drugs such as β -lactam antibiotics,¹⁶ penicillin, and cephalosporins, have two peptide bonds and a carboxyl group. Some β -lactam antibiotics also contain amino groups such as cephalexin and cefadroxil, which are absorbed well after oral administration (Figure 16.5). Most β -lactam antibiotics have no free amino group and exhibit negligible oral bioavailability. Cefixime, which has no amino group but has two carboxyl groups, is absorbed with intermediate oral bioavailability, presumably mediated by PEPT1 (Figure 16.5). So, amino group and carboxyl group may not be essential moieties for PEPT1 substrates. Furthermore, as shown in Figure 16.4, some compounds like valacyclovir has no peptide bond, but are transported by PEPT1, suggesting that peptide bond may not be prerequisite to be transported by PEPT1. So, now it is thought that PEPT1 has broad substrate selectivity than expected from the native substrates. This observation suggests that PEPT1 is useful for the improvement of intestinal membrane permeability of peptide-like drugs and/or drug candidates.

In order to find which drugs or drug candidates are substrates of transporters, screening is usually done by in vitro-cultured cells that express the target transporters. However, it is often observed that PEPT1 substrates



Structures of PEPT1 Substrates, Dipeptides and B-Lactam Antibiotics

FIGURE 16.5 Structures of PEPT1 substrates, dipeptides and β -lactam antibiotics. Typical substrates of PEPT1 have peptide bond, carboxyl terminals and amino terminals within the molecules.



FIGURE 16.6 pH Dependence of PEPT1 activity for various substrates studied in *Xenopus* oocytes expressed with human PEPT1. Anionic FK089, neutral glycylsarcosine, and cationic carnosine are all substrates of PEPT1 when examined by *Xenopus* oocytes that are injected with cRNA of PEPT1 and cultured for a few days to express matured PEPT1 proteins. Closed and open columns represent the result of PEPT1-expressed (injected with cRNA of PEPT1) and not expressed (injected with water), respectively. Transport activities for each substrate exhibit variable pHprofiles with differential optimal transport pH depending on the substrates.

identified in vitro do not necessarily exhibit high oral bioavailability after in vivo oral administration. For example, cefadroxil exhibits a high bioavailability after oral administration, while cefixime does not exhibit high intestinal absorption. Such differences in the bioavailability among PEPT1 substrates may be explained by the fact that the physiological conditions, especially pH, are not necessarily optimal for all the substrates to be transported by PEPT1 in the intestinal lumen in vivo. Cefixime and FK089 have very similar structures and are substrates of PEPT1 (Figure 16.5). They have two carboxyl groups and are classified as anionic β -lactam antibiotics. In the case of FK089, it shows a different transport-pH profile from zwitterionic cefadroxil (Figure 16.6). Figure 16.6 shows pH-profiles of transports by PEPT1 of three typical substrates, including anionic (FK089), neutral (glycylsarcosine), or cationic peptides (carnosine). By the in vitro PEPT1-mediated transport studies, FK089 showed higher transport at pH 5 with a lower activity at pH 6 and 7, whereas cefadroxil exhibited optimal pH at around 6. In the case of carnosine, optimal transport by PEPT1 is shifted to pH 7 and lowest activity at pH 5 (Figure 16.6). This observation demonstrated that although PEPT1 is energized by proton gradient, the optimal activity is not necessarily highest at acidic pH and is variable among the substrates. Accordingly, the intestinal luminal pH should largely affect the transport activity of PEPT1, depending on the pH profiles of the substrates. Thus, it would be possible to improve the intestinal membrane permeability of PEPT1 substrates that exhibit a low oral bioavailability by optimizing the intestinal luminal pH as required.

16.3.3 INTESTINAL REGIONAL DIFFERENCE OF ABSORPTION OF PEPT1 SUBSTRATES

Intestinal transport of a β -lactam antibiotic cefadroxil, a substrate of PEPT1 shown in Figure 16.5, is variable among intestinal regions from duodenum (upper part of small intestine) to ileum (lower part of small intestine), depending on the method used for evaluation of small intestinal absorption.¹⁷ Expression of PEPT1 mRNA in ileum is the highest and is the lowest in duodenum of rat small intestine, but the absorption rate of cefadroxil from the ileum was the lowest when absorption is evaluated by the in situ intestinal closed-loop method in rats (Figure 16.7).¹⁷ On the other hand, when intestinal membrane permeability was evaluated using isolated intestinal tissues with Ussing chamber method, the permeability of cefadroxil correlates well with the expression level of PEPT1 along the small intestine.¹⁷ This apparent discrepant observation was explained by the pH in the intestinal lumen. When intestinal absorption was studied by the in situ loop method, the pH in ileum loop was increased from pH 6.0 (pH of the administered cefadroxil buffer solution) to alkaline (pH 7.9), whereas pH values in the duodenum (upper part) and jejunum (middle part) were changed slightly to higher pHs of 6.5 and 6.3, respectively (Figure 16.7). Physiologically, luminal pH of the upper part



FIGURE 16.7 Regional difference of cefadroxil absorption by in situ small intestinal loop method in rats. Intestinal absorption of cefadroxil was evaluated by in situ loop method of upper (duodenum), middle (jejunum) and lower (ileum) part of the small intestine in rats. Cefadroxil was administered as the buffer solution adjusted to pH 6.0 into each loop, and the remaining cefadroxil solution in each loop was collected 20 min after administration. The amount of cefadroxil remaining and the pH of the collected solution were measured. The ordinate represents the disappeared amount of cefadroxil from the loop (absorbed % of dose) the number in each bar represents the pH values of the recovered solution from each loop. Ileum exhibited lowest absorption and highest pH.

of small intestine is acidic, while pH at the lower part of small intestine is high, presumably owing to secretion of bicarbonate or other alkalinizing agents from the epithelial cells into the lumen. On the other hand, when intestinal absorption was evaluated in vitro by Ussing chamber method, large volume of the test solution used enabled to minimize the effect of alkalinizing agents during the absorption study. This observation demonstrated that the intestinal luminal pH is important to determine the apparent membrane permeability via PEPT1.

16.4 MANIPULATION OF INTESTINAL LUMINAL pH BY BIOMATERIALS AND IMPROVEMENT OF INTESTINAL ABSORPTION VIA PEPT1

16.4.1 Selection of Biomaterials to Maintain Lower pH in Intestinal Lumen

In order to demonstrate that lowering of the intestinal luminal pH improves the PEPT1-mediated permeability of peptide-mimetics, a pH-dependent anionic polymer, Eudragit L100-55, was selected. Eudragit is used commonly for enteric coating of oral formulations.¹⁸ There are many kinds of Eudragit polymers in the market from Degussa Corp. (Piscataway, NJ; http://www. roehm.com). Eudragit is characterized as an anionic polymer of methacrylic acid and methacrylates and contains a carboxyl group as a functional group (Figure 16.8). These polymers are not dissolved under acidic condition, and



FIGURE 16.8 Structures of proton releasing Eudragit 1100-55 and proton nonreleasing Eudragit RSPO. Eudragit L100-55 has free carboxyl groups within the molecule that enables the pH of the solution to be acidic. Eudragit RSPO has no ionizable group and has no effect on pH.



FIGURE 16.9 Effect of pH on the PEPT1-mediated transport of cefadroxil and cefixime (A) and polymer-concentration dependence of the pH (B). PEPT1-mediated transport of cefadroxil and cefixime was evaluated by Caco-2 cells that express PEPT1. Initial uptakes of both compounds were measured at various pH values, showing different optimal pH. (B) Effect of increasing concentrations of Eudragit L100-55 and Eudragit RSPO on the pH of the buffer solution, initially adjusted to pH 6.0, were measured. Eudragit L100-55 can acidify the buffer solution to optimize the transport of cefixime by PEPT1, while Eudragit RSPO does not affect pH at all.

become soluble and release protons under weakly acidic pH to alkaline condition due to its carboxyl groups, thereby controlling the intestinal luminal pH to be acidic. Eudragit products have negligible toxicity to the intestinal tissues according to the manufacturer's information. Furthermore, related Eudragit products such as neutral polymers are also available, which can be used as the pH-insensitive control polymers. In the present study, Eudragit L100-55 and Eudragit RSPO (aminoalkyl methacrylate copolymer) shown in Figure 16.8, were used as the acidic and neutral polymers, respectively, since Eudragit L100-55 dissolves at pH 5.5 and can maintain pH of the solution acidic, while Eudragit RSPO does not affect pH of the solution (Figure 16.9B). By using these polymers, the present strategy to activate PEPT1-mediated transport of peptide-mimetics by optimizing pH in the lumen was examined.

16.4.2 SELECTION OF TEST COMPOUND FOR PEPT1-MEDIATED ABSORPTION

 β -lactam antibiotics were selected as a test compound to study the effect of PEPT1 activation by acidic polymers, since there are many derivatives with

variable oral bioavailability and the assay of the plasma concentration is feasible. Cefadroxil (Figure 16.5) exhibits almost complete absorption after oral administration and is a good substrate of PEPT1. Optimal pH for the transport of cefadroxil by PEPT1 is around pH 6, since it is classified as a neutral peptide by having both of carboxyl- and amino-terminals (Figure 16.9). Since cefadroxil has a high oral bioavailability, it is difficult to observe an increment of oral bioavailability by the polymers. Cefixime (Figure 16.6), which is classified as an anionic peptide with two carboxyl groups, is a substrate of PEPT1 at acidic pH as shown in Figure 16.9. However, it exhibits low bioavailability, obtained as the ratio of AUCs in plasma concentrations after oral administration and intravenous administration, and is less than 30% in rats (Figure 16.10). Since cefixime transport by PEPT1 significantly increases with decrease in pH to acidic (Figure 16.9), it should be a good test compound for the present study.

Figure 16.11 illustrates the concept and strategies to improve the intestinal absorption of cefixime, a peptide mimetic drug, using acidic polymers. PEPT1 expression in distal small intestine is higher than proximal part and pH in the distal part is higher than proximal part. The absorption of peptides/ peptide-mimetics in proximal part is higher than the distal part, suggesting that pH significantly affects PEPT1 activity. Accordingly, it is hypothesized that by lowering pH using acidic polymer such as Eudragit L100-55, intestinal absorption of low permeable cefixime could be improved.



FIGURE 16.10 Plasma concentration of cefixime after oral and bolus IV administrations in rats. Cefixime solution was administered to rats orally and intravenously and the plasma concentration of cefixime was measured for 8 hr. Absolute bioavailability (BA) was evaluated by the ration of AUCpo/AUCiv. Cefixime is absorbed after oral administration with a low bioavailability, 27.1%.

Improvement of Intestinal absorption of Cefixime by Activation of PEPT1 using Acidic Polymer Eudragit L100-55



FIGURE 16.11 Scheme for the improvement of intestinal absorption of cefixime by activation of PEPT1 using acidic polymer Eudragit L100-55. By supplying protons by Eudragit L100-55 to intestinal lumen, especially lower part of the small intestine, where physiological pH is too high to activate PEPT1 but expresses large amount of PEPT1, membrane permeability of cefixime can be improved.

16.4.3 IMPROVEMENT OF INTESTINAL ABSORPTION OF CEFIXIME BY EUDRAGIT L100-55

First of all, the usefulness of Eudragit L100-55 was examined by in situ loop method in rats. The effect of increasing concentrations of the acidic polymer on the absorption of cefadroxil and cefixime in shown in Figure 16.12. pH of the recovered solution in the lumen of ileum 20 min after administration decreased in the presence of the polymer (Figure 16.12C,D). With decrease in pH, absorption of cefadroxil and cefixime was increased, when evaluated by in situ closed loop method of rat ileum (Figure 16.12A,B). Here, the extent of increment of absorption by Eudragit L100-55 was more significant in cefixime than cefadroxil, since cefixime exhibited lower absorption when the polymer was not included. These results demonstrated that the strategy using acidic polymer Eudragit L100-55 to improve intestinal membrane permeability of cefixime mediated by PEPT1 is feasible. Accordingly, in vivo intestinal absorption of cefixime was evaluated by coadministration of the polymers in rats. Based on the result of Figure 16.12 and the practical handling, 5 w/v% concentration of Eudragit L100-55 was selected for the following in vivo studies.



FIGURE 16.12 Influence of Eudragit L100-55 on intestinal pH and absorption of cefixime and cefadroxil in rat ileal loops. Intestinal absorption of cefadrocil (A) and cefixime (B) were evaluated by in situ ileal loop method in the presence of Eudragit L100-55. By adding Eudragit L100-55, pH of the administered solution was decreased (C,D) and the absorption was increased with the decrease of pH.

After oral administration of cefixime solution to rats with or without suspended Eudragit L100-55, plasma concentration of cefixime was measured (Figure 16.13). When cefixime was coadministered with Eudragit L100-55, plasma concentration of cefixime was higher than those without Eudragit L100-55. However, when neutral polymer Eudragit RSPO was coadministered with cefixime, no significant change in plasma concentration of cefixime was observed. The evaluated pharmacokinetic parameters for these results are summarized in Table 16.1. Absolute oral bioavailability was significantly increased from 27.1 to 62.2% by coadministrating Eudragit L100-55, while such an increase was not observed by Eudragit RSPO. A slight increase in T_{max} in the presence of Eudragit polymers may suggest the decreased transit rate through the intestinal lumen, because the polymers were administered in suspension. Since no significant change was observed in the elimination rate constant k_e and the neutral polymer did not improve AUC or C_{max} , the apparent increase in AUC and C_{max} by Eudragit L100-55 is due to the increased absorption, presumably by the increased membrane permeability by lowering pH optimal for cefixime.

Figure 16.14 shows the effect of PEPT1 inhibitor on the absorption of cefixime. Here, cefadroxil was used as a PEPT1 inhibitor at the concentrations of 2 and 10 mM, since the $K_{\rm m}$ for cefadroxil to PEPT1 is around 2 mM. When 10 mM cefadroxil was included in the cefixime solution with Eudragit L100-55, plasma concentration was decreased significantly, and with 2 mM cefadroxil

Effect of Eudragit L100-55 and RSPO on Plasma Concentration of Cefixime after Oral Administration to Rats



FIGURE 16.13 Effect of Eudragit L100-55 and Eudragit RSPO on plasma concentration of cefixime after oral administration to rats. Cefixime solution was orally administered to rats with or without Eudragit polymers in suspension. Plasma samples were obtained for 8 hr. By coadministrating cefixime with acidic polymer Eudragit L100-55, significant increase in plasma concentration was observed, while no change was observed by neutral Eudragit RSPO.

less but significant decrease in plasma concentration of cefixime was observed (Figure 16.14). The evaluated pharmacokinetic parameters in the presence of cefadroxil were also summarized in Table 16.1. In the presence of 10 mM cefadroxil, $C_{\rm max}$ and AUC of cefixime in the presence of Eudragit L100-55 was decreased to be almost comparable values in the absence of Eudragit

TABLE 16.1 Pharmacokinetic parameters of cefixime after oral administration							
		+EL	+ER	+CDX(10)+EL	+CDX(2)+EL	IV	
n	8	5	4	7	3	4	
$C_{\rm max} ~(\mu g/mL)$	4.1	11.5*	3.4	3.4†	6.4		
$T_{\rm max}$ (hr)	0.7	1.2	1.0	1.4	1.0		
ACU_{∞}	12.6	29.0*	9.1	12.8†	26.8	46.6	
Absolute BA (%)	27.1	62.2*	19.6	27.5†	57.6		
$k_{\rm e} ({\rm hr}^{-1})$	0.35	0.37	0.61	0.38	0.34	0.51	

*Significantly different from the control values (p < 0.05).

†Significantly different from the +EL values (p < 0.05).

EL, Eudragit L100-55; ER, Eudragit RSPO; +CDX(10), with 10 mM cefadroxil; +CDX(2), with 2 mM cefadroxil; IV, intravenous administration; C_{max} , maximum plasma concentration; T_{max} , Time of C_{max} ; BA, bioavailability; k_e , elimination rate constant.



FIGURE 16.14 Inhibitory effect of cefadroxil on plasma concentration of cefixime after oral administration to rats. Cefixime was orally administered to rat with Eudragit L100-55 in the presence or absence of cefadroxil at 2 mM or 10 mM. Increased plasma concentration of cefixime by coadministration of Eudragit L100-55 was lowered by high-concentration of cefadroxil (10 mM), showing that increased cefixime concentration by Eudragit L100-55 is due to activation of PEPT1, since cefadroxil is a stable inhibitor of PEPT1.

L100-55. These concentration-dependent effect of cefadroxil strongly demonstrated that the mechanism of an increase in the absorption of cefixime by acidic polymers is due to the activation of PEPT1 by lowering pH, which is optimal for cefixime transport.

16.5 CONCLUDING REMARKS

The strategy shown in this chapter to enhance the absorption of cefixime via PEPT1 by pH-regulating biomaterials is applicable to other drugs or drug candidates that are substrates of PEPT1. This method is useful, because an improvement in oral bioavailability is possible without further chemical modification of the drug molecules such as prodrug strategy. Until now, it was believed that increase in membrane permeability is impossible without modification of the chemical structures. So, this is the first example that pharmaceutical formulation technology using biomaterials can be used to improve membrane permeability via the intestinal transporter. This idea is based on the precise characterization of PEPT1 transporter, suggesting that basic information on the membrane transporters will be useful for the practical application to drug delivery. It is well known that some transporters other than peptide transporters also utilize the proton gradient as the driving force. Monocarboxylic acid transporter MCT1 transports monocarboxylic acids coupled with a proton flux across the cell membrane.^{19,20} D-Cycloserine is transported by a proton-coupled amino acid transporter.²¹ Organic anion

transporter OATP-B is expressed at the apical membrane of human small intestinal epithelial cells and exhibits pH-dependent transport activity.^{22,23} Thus, the same strategy should be applicable to drugs that are substrates of these transporters, but are not absorbed well *in vivo*. In addition, membrane permeation via simple diffusion according to pH-partition hypothesis can also be manipulated by the acidic polymers, since pH also affects diffusional permeability due to pH-partition hypothesis. Indeed, intestinal permeability of an anionic drug furosemide was improved in some extent.²⁴

Usually polymers are not absorbed well from the gastrointestinal tract because of their large molecular weights. Accordingly, if direct toxicities to the intestinal tissues is not anticipated by the applied polymers, they can be used to manipulate drug dispositions in the gastrointestinal tract with minimum systemic influence. Further understanding of the intestinal membrane transport mechanisms of drugs will enable improvement of the membrane permeability by optimization using biomaterials, which often limits the development of orally active drugs. Finally, the results of this chapter have been reported and the precise experimental conditions can be obtained from the original article.²⁵

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17 Protein Transduction Domain as a Novel Tool for Delivery of Proteins, Peptides and Nucleic Acids

Ricardo L. Pastori, Melina M. Ribeiro, Dagmar Klein, and Camillo Ricordi

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Acknowledgments

References

17.1 INTRODUCTION

Proteins have a primordial role in mammalian cell functions and are the most abundant cellular macromolecules. They have a key role in cellular structure, regulation of signal transduction, discharge of genetic information and cellular metabolism. Therefore, delivery of proteins and peptides into cells would be an extremely valuable approach for therapeutic purposes as well as for cell biology studies.

The cell membrane restricts protein and peptide translocation into the cytoplasm and so limits their use as pharmaceutical and research tools. The phospholipid bilayer structure of the cell membrane efficiently controls inward and outward flow of molecules. Only gases and a few small molecules are able to passively diffuse through the cell membrane. Internalization of other proteins and peptides is severely restricted by their size and polarity.¹

Several methods for introduction of proteins/peptides into cells are explored and described in the literature. The most studied and utilized are electroporation, microinjection, entrapment within liposomes and polymeric delivery systems.^{2–4} Nonetheless, factors such as toxicity, limited cell transduction efficiency and reproducibility have restricted their application.

A rapidly evolving technology known as protein transduction has been recently developed to deliver proteins and peptides into cells and tissues.⁵ This technology is based on the ability of certain small peptides known as protein transduction domains (PTD) to cross the cell membranes. Thus, fusing proteins or peptides to PTDs, either chemically or genetically, allows direct delivery of the PTD-fused molecules into cells. The most studied and described PTDs originate from naturally occurring proteins. However, synthetic PTDs have been recently developed as well.

PTDs can be divided into three groups based on their structures: the first group consists of PTDs with cation residues as a common feature, especially with arginines, that have a key role in transduction of PTD-fused molecules. The second group is PTDs that have an amphiphilic or amphipathic structure with hydrophobic and positively charged residues in opposite sides of the structure. The third group is of non-polar PTDs that are derived from the hydrophobic core region of peptide leader sequences, of secreted or cell surface proteins. Although the detailed mechanism of cellular uptake is still not well known, it is well possible that PTDs differ in their mechanism of internalization. Several in depth, comprehensive reviews have been published on this subject.^{5–7} Table 17.1 compiles PTDs, derived from naturally occurring proteins as well as synthetic PTDs that have been characterized and described in the literature.^{8–22} We will now focus on molecular description and protein/ peptide/nucleic acid transduction capabilities of the most researched and best-characterized PTDs.

17.2 PTDs DERIVED FROM NATURALLY OCCURRING PROTEINS

The most extensively characterized PTDs in this group are the transactivating transcriptional transactivator (TAT) protein (86-mer polypeptide) of the human immunodeficiency virus-1 (HIV-1/TAT), *Drosophila*–Antennapedia transcriptional factor (ANTP) and VP22 transcriptional factor from herpes simplex virus type-1 (HSV-1).

TABLE 17.1List of naturally occurring and synthetic protein transduction domains (PTDs)

Protein/PTDs name	Sequence	Reference
HIV-TAT/PTD	YGRKKRRQRRR	8,9
Antennapedia/ Penetratin	RQIKIWFQNRRMKWKK	10
PDX-1/	RHIKIWFQNRRMKWKK	11
HSV-VP22/	NAATATRGRSAASRPTERPRA PARSASRPRRPVE	12
Protegrins/Syn B1	RGGRLSYSRRRFSTSTGR	13
Kaposi FGF/MTS	AAVALLPAVLLAAP	14
HBV-PreS2/TLM	PLSSIFSRIGDP	15
Vascular endothelial cadherine/pVec	LLILRRRIRKQAHAHSK	16
Prion protein	N-terminal (1–28)	17
Synthetic PTDs		
PTD name	Sequence	Reference
PTD-4	YARAAARQARA	18
PEP-1	KETWWETWWTEWSQPKKKRKV?	19
PTD-5	RRQRRTSKLMKR	20
Transportan	GWTLNSAGYLLGKINLKALAA LAKKIL	21
PolyArginine	RRRRRR	22
Polyguanidine peptoids	7 to 9 guanidine head groups of arginine on a glycine backbone.	22

PTDs from naturally occurring proteins

17.2.1 HIV-1/TAT PROTEIN

The first two pioneer studies reporting the transducing capabilities of the HIV/TAT protein were published back to back more than fifteen years ago in 1988.^{23,24} Subsequently, heterologous proteins fused to the TAT protein, either chemically or generated as a recombinant protein, were delivered into cells.^{25,26} Recently, Dowdy et al. have developed a bacterial expression-based system to produce proteins fused to residues 47–58 (TAT/PTD). This is the TAT protein basic domain, rich in arginine residues, that constitutes the minimal sequence required for transduction.^{8,9} The prokaryotic expression vector contains the TAT/PTD-fusion protein under the control of bacteriophage T7 promoter (Figure 17.1). This method allows for the generation of sufficient amount of TAT/PTD-fusion recombinant proteins are capable of transducing wide variety of cells and tissues including the hemato-encephalic barrier, which shows resiliency to most methods of exogenous molecules introduction.²⁷ However transduction of TAT/PTD derivative molecules is not without restrictions. Indeed, some tissues or cells, such as bladder epithelial cells cannot



FIGURE 17.1 Generation of TAT/PTD-fusion recombinant proteins. The bacterial expression cassette of TAT/PTD fusion proteins consists of the ATG (Metionine) initiator codon and a N terminal stretch of six histidine residues to allow for binding to a Ni^{2+} column and purification of the fusion protein. The 11-amino-acid TAT protein transduction domain (in bold) contains a potential nuclear localization signal (underlined).

be transduced.²⁸ Similarly, binding of TAT/PTD fusion proteins to extracellular matrix components can interfere with their cell transduction capability.²⁹

It has been historically argued that the process of translocation of peptide/ proteins fused to TAT/PTD into cells, known as transduction, was independent of cellular receptors and of temperature, suggesting that energydependant endocytosis was not involved. This concept has been now disproved as a cell fixation artifact³⁰ and several groups have recently shown that cargo molecules fused to TAT/PTD enter cells through endocytosis mechanism.³¹⁻³⁴ Collectively, new investigations suggest that a first step in the process of transduction is binding of the positively charged TAT/PTD to negatively charged components of the cell membrane. Substitution of the positively charged arginine residues by alanines completely inhibits transduction capability.²² Subsequently, the TAT/PTD fusion molecule enters into cells via energy-dependent endocytosis (Figure 17.2). Both, conventional clathrinendocytosis and nonclassic caveolar endocytosis have been reported taking place in the translocation process. In the first type of endocytosis the TAT/ PTD derivative is internalized in a clathrin-coated pit. Caveolae endocytosis, on the other hand, utilizes caveolae, 50-80 nm plasma membrane invaginations, rich in cholesterol and sphingolipids.³⁵ Once the caveolae endosome is formed the TAT/PTD molecule is internalized in the cytoplasm. Regardless of the endocytosis mechanism used for translocation, it has been suggested that endosome or caveolosome internalized TAT/PTD must be released into the



FIGURE 17.2 Transduction of cells by the PTD from the HIV-1/TAT protein. Protein/ peptides fused to TAT/PTD are represented as cargoes linked to dots. The mechanism of entrance of TAT/PTD fusion proteins involves endocytosis followed by a subsequent escape from the endocytosis vesicle, after which the fused protein could stay in the cytoplasm or could go to the nuclei. It is believed that the first step involving the interaction of the positively charged TAT/PTD with negative cell membrane environment has an important role in the translocation process. TAT/PTD fusion proteins can be targeted to specific organelles. For example, TAT/PTD engineered with a mitochondrial localization signal (MLS) enters the mitochondria and is recognized and cleaved. The fused protein cargo stays anchored in the mitochondria.

cytoplasmic compartment in order to achieve full biological function (Figure 17.2). The subcellular location of transduced protein depends on the cell type, nature of the protein, and method of delivery.³⁶

Wide variety of biologically active peptides and full-length proteins, of molecular weight up to 120 kDa, have been delivered to cells, tissues and organs in vitro, ex vivo and *in vivo*,^{5,6,37} offering exciting therapeutic applications. For example, *in vivo* administration of the anti-apoptotic member of Bcl2 protein family, Bcl-XL confers neuronal protection and reduces ischemia brain damage.^{38,39} Furthermore, TAT/PTD-fusion proteins have been generated to specifically target cell organelles. For instance, engineering

a mitochondrial recognition sequence (MRS) between the protein cargo and the TAT/PTD allows for anchoring proteins in the matrix of mitochondria. Once the PTD-fusion protein is transduced into the cell and specifically into mitochondria, the MRS is cleaved away from the fusion protein resulting in transduced protein being anchored in the mitochondria (Figure 17.2). TAT/ PTD-MRS-fusion protein has remarkable property. When administered into pregnant mice the fusion protein is capable of crossing the placenta and localizing in both the fetus and the newborn pups.⁴⁰ Schwarze et al. (1999)²⁷ demonstrated that the intraperitoneal injection of TAT- β -galactosidase into mice results in detectable β -gal activity in bone marrow as well as in all regions of the brain. Moreover, TAT- β -gal did not disrupt the blood-brain barier as assayed by co-injection with Evan's blue dye.

It has not been determined yet what size of molecules can be efficiently cargoed by TAT/PTD. Among others TAT protein transduction technology has been applied to introduce ions, such as ^{99 m}Tc. This may be applied in imaging medicine.⁴¹ Additionally, TAT/PTD has been successfully used to deliver macromolecules that could be used for therapeutic purposes.^{42,43} In it allowed delivery of N-(2-hydroxypropyl)methacrylamide particular. (HPMA) copolymer to both cytoplasm and nuclei of human ovarian carcinoma cells.⁴³ This technology holds great promise for the development of polymer-based systems for the delivery of therapeutic molecules. Using TAT protein supramagnetic particles and nanostructures were delivered into cells.44,45 Supramagnetic particles fused to TAT/PTD were delivered to hematopoetic cells in order to investigate homing profile of leukocytes in vivo and to develop new pharmacological transporters.⁴⁴ The surfacefunctionalization of shell cross-linked nanoparticles with the TAT/PTD peptide sequence YGRKKRRQRRR allowed cell binding and internalization of nanobioconjugates to CHO and Hela cells.45

TAT delivery system is particularly useful when the expression of a particular gene is only transiently needed as it often happens in cell transplantation. For example, due to the development of new immunosuppressive regimens, transplantation of pancreatic islets has become a therapeutic treatment for type I diabetes.⁴⁶ However, despite the great advances achieved in islet technology, a substantial number of islets die during culture due, partly, to programmed cell death, particularly apoptosis. Pancreatic islets are efficiently transduced by TAT protein transduction domain (Figure 17.3). We tested the transduction capability of TAT/PTD in live nonfixed islets to avoid potential fixative artifacts. Human pancreatic islets were transduced with TAT/PTD labeled with fluorescein isothiocyanate (FITC). Transduction was assessed by confocal microscopy in nonfixed, live islet cells, and propidium iodide staining excluded dead cells. Images were collected on five different focal planes demonstrating the ability of TAT protein domain to transduce throughout the entire islet. Furthermore, transduction of islet with antiapoptotic proteins can significantly enhance the viability of islets in culture with the advantage that islets remain genetically unmodified, thus avoiding undesirable long-term effects.47,48



FIGURE 17.3 (See color insert following page 512) Transduction of pancreatic islets in culture with TAT/PTD. Sectional scanning using confocal microscopy of live non-fixed islets. To test the transduction capability of TAT/PTD, human pancreatic islets were transduced with a 14-mer TAT peptide control labeled with fluorescein isothiocyanate (GYGRKKRRQRRRGC-FITC). In order to avoid potential fixation artifacts, transduction was analyzed by confocal microscopy in non-fixed live cells. Islets were simultaneously stained with propidium iodide to rule out false positives (membrane damaged dead cells).

17.2.2 HOMEOBOX PROTEINS

Homeobox proteins are a family of transcription factors which have a key role in regulation of many developmental genes. The homeobox or homeodomain that characterizes the members of this family is a region of 60 aa forming three alpha-helixes connected by β -turns. Prochiantz's group first reported that the entire homeodomain of the Drosophila-Antennapedia transcription factor, was internalized by cells in culture.⁴⁹ The same group, later found that the16 aa, of homeodomain's third α -helix, (residues 43–58) (RQIKIWFQNRRMKWKK) known as penetratin, had the same capability as the entire homeodomain.⁵⁰ Similar to TAT/PTD, the mechanism of cell membrane translocation is currently unknown. Previous studies performed on artificial phospolipid bilayers suggested a possible mechanism of translocation involving formation of inverted micelles.¹⁰ However, recent work performed on live, nonfixed cells indicated, that similar to TAT/PTD, penetratin could enter into cells via endocvtosis-like mechanism.⁵¹ Penetratin has been mostly applied to delivery of small peptides. Table 17.2 shows peptides that have been delivered in vivo via penetratin, that could have therapeutic potential.⁴⁶⁻⁴⁹

TABLE 17.2In vivo delivery of protein/peptides through penetratin

Protein/peptide	Description and potential application		
P16	The administration of penetratin fused to a 20 amino acids peptide (aa 84–103) from p16 tumor suppressor protein suppressed pancreatic cancer growth and extended survival in mice. ⁴⁶		
α-smooth muscle actin	The NH ₂ -terminal sequence EEED of α -smooth muscle actin, is a marker of fibroblast–myofibroblast modulation. Topical administration of Penetratin–EEED inhibits the contraction of rat wound granulation tissue. ⁴⁷ This approach could help to develop new therapeutic strategies for fibrocontractive pathology.		
Caveolin	The scaffolding domain of caveolin-1, primary coat protein of caveolae endosome, fused to penetratin successfully suppressed acute inflammation and vascular leak in mice. This could have potential to develop new therapeutic approach against endothelial inflammation. ⁴⁸		
NEMO	An amino terminal-helical region of NEMO (Nuclear factor kappa B essential modifier) containing the NEMO binding domain fused to penetratin inhibited inflammatory responses in two experimental mouse models of acute inflammation. ⁴⁹		

Other homeobox transcription factors have also been reported to carry a protein transduction capability sequence embedded in their homeodomain:

- Fushi-tarazu and Engrailed, two *Drosophila* homeodomain proteins have similar transduction properties.⁵²
- the rat protein islet-1, an insulin enhancer containing pIsl1, a PTD encompassing amino acid residues 45–60 (RVIVWFQNKKRCKDKK) of the third helix motif.⁵³
- PDX-1 (pancreatic and duodenal homeobox-1), transcription factor that regulates insulin transcription and has a critical role in pancreatic development, contains penetratin-like PTD, (RHIKIWFQNRRMKW KK), in the homeodomain's third α -helix and can be delivered in vitro to pancreatic ducts and islets.¹¹
- HOXB4, the third helix of human homeobox B4 protein, contains the sequence identical to the Antennapedia-PTD. Human stem cells, cultured on stromal cells genetically engineered to secrete HOXB4, have been expanded by more than 20-fold over their input numbers. This expansion was associated with enhanced stem cell repopulating capacity *in vivo* and maintenance of pluripotentiality.⁵⁴
17.2.3 HSV/VP22 PROTEIN

It has been reported that the translocation capability of the 38 kDa structural protein from the herpes simplex virus was based on the entire protein. Unlike the other PTDs, VP22 did not have a shorter domain capable of cellular transduction. A unique feature of VP22 is the capability to mediate intercellular trafficking. Most of the published literature comprises studies in which the production of VP22 fused protein was genetically engineered and diffused to nontransfected neighboring cells via intercellular trafficking. However, a recent study showed that VP22 C-terminal 34 amino acids were sufficient for import of proteins into cells.¹² In vivo application of transduction via intercellular trafficking of VP22-fused derivatives was achieved with tumor suppressor p53^{55,56} and the enzyme thymidine kinase.⁵⁷ VP22-protein transduction was reported to be successful in the delivery of therapeutic proteins into cells of the central nervous system.⁵⁸ VP22 protein translocation capability has also been utilized through a rather unusual technology. The soluble subdomain of VP22 (residues 105-300) induces formation of spherical particles when incubated with small oligonucleotides. The particles termed vectosomes are taken up by a number of cell types, remain in the cytoplasm for several days until disrupted by light activation. Using this phenomenon a short peptide originating from pro-apoptotic BH3 domain of Bak protein was delivered into cells via vectosomes and subsequently released into the cytoplasm by light activation 59

17.2.4 OTHER PTD DERIVED FROM NATURALLY OCCURRING PROTEINS

A group of less characterized and documented PTDs originating from natural occurring proteins includes PreS2 protein¹⁵ from hepatitis B virus, membrane translocation sequence (MTS) from Kaposi fibroblast growth factor¹⁴ and peptide SynB1 from protegrins family of peptides.¹³ PreS2 protein expressed on the surface of hepatitis B virus (HBV) contains amphipathic peptide residues 41–52 with membrane translocation properties. The peptide was used to express the HBX regulatory protein from HBV.⁶⁰ The nonconserved hydrophobic region of signal peptides has the capability of crossing cell membranes. In particular, the 12 residues of Kaposi's fibroblast growth factor, a hydrophobic domain, termed MTS, has been utilized to deliver several peptides and proteins.¹⁴

The SynB1 peptide is derived from protegrins, a family of natural small peptides with antimicrobial capability.⁶¹ It has an amphipathic structure, capable of crossing cell membranes and blood–brain barrier without lytic activity. It has also been used to deliver anti-neoplastic drugs into brain. For instance, SynB1 coupled to doxorubicin significantly enhances the extent of doxorubicin penetration through the blood–brain barrier.¹³

17.3 SYNTHETIC PTDs

Reflecting the significant increase in number of laboratories interested in studying delivery of proteins and peptides through PTDs, several synthetic PTDs have been recently reported. The most promising PTD designed rationally to increase their cell translocation capabilities are:

- *PTD-5*. This protein transduction domain was discovered by screening an M13 phage display library for cationic peptides. PTD-5 has a high content of Arg residues (Table 17.1). Fused to an antimicrobial peptide induced apoptosis and tumor reduction of human head and neck tumor in mice.⁶² In addition, when fused to PTD5 a peptide derived from NEMO protein (nuclear factor kappa B essential modifier) inhibited activation of nuclear factor kappa B in pancreatic islets *in vivo*.⁶³
- *PTD-4*. Based on the observation that the arginine-rich TAT/PTD has a strong amphipathic structure, a synthetic peptide known as PTD-4 was designed to acquire an optimized amphipathic structure with arginine residues alignment on one face of the helix and an Ala residues to strengthen the alpha-helix structure of the peptide on the other.¹⁸ PTD-4 was utilized to deliver the SH3 domain of an adapter protein.⁶⁴
- *Polyarginine and polyguanidine peptoid derivatives*. Arginine residues not only have a critical role in the transduction capability of TAT/PTD, but was also shown to have a polyarginine containing seven arginine residues (R7) that was more efficient in translocating through cell membranes.²² Moreover, it was observed that the side chain guanidine group from arginine residues, was ultimately responsible for cell transduction.²² Based on this fact an efficient protease-resistant molecular transporters consisting of polyguanidine peptoid derivatives were developed.²²
- *Pep-1*. This is a short amphipathic peptide engineered by combining three domains: a hydrophobic-tryptophan rich domain required for efficient transduction and interaction with protein hydrophobic pockets, a separator domain and a nuclear localization signal domain from SV-40 T-large antigen. Its main feature is the capability of delivering proteins that are not covalently bound.¹⁹ A mouse emphysema model was generated by delivery of active caspase-3 *in vivo* by PEP-1.⁶⁵

17.4 PROTEIN TRANSDUCTION DOMAINS FOR DELIVERY OF NUCLEIC ACIDS

Gene therapy is presently considered to be a promising way to cure a broad spectrum of diseases. Because of low biomembrane permeability and rapid degradation of DNA and oligonucleotides, they are believed to have limited therapeutic value of their own. Although viral gene transfer technologies have greatly improved during the last decade, basic concerns regarding their safety still remain. Limited targeting of cells, integration with potential oncogenes and unwanted immunoresponse are the greatest drawbacks of this method. Therefore, PTD-mediated delivery of genes could potentially offer new possibilities for gene therapy in medical research.⁶⁶

17.4.1 DELIVERY OF DNA

PTDs are efficient DNA condensating agents that protect it from anionic proteoglycans disruption and degradation,⁶⁷ allowing for intracellular transport across mammalian and prokaryotic cells in vitro and *in vivo* with no toxic effect. However, methods that do not involve condensation can also be used for DNA delivery. For example, engineering an λ phage to display the TAT peptide on its surface, greatly facilitates transfection of encapsulated DNA into mammalian cells.⁶⁸

Several results indicate that TAT peptide may become a useful component of synthetic gene delivery vehicles, applicable in the *in vivo* transfer of therapeutic genes. In order to facilitate membrane transduction TAT peptides can be bound directly to DNA or to DNA carriers, or even be just present in the DNA mixture. For this purpose different strategies have been described. Electrostatic based complexes of plasmid DNA with monomeric² or oligomeric TAT peptides⁶⁹ (2 to 4 molecules) facilitated the transduction into mammalian cells and expression of genes. Similarly, branching TAT peptides, containing eight TAT moieties were able to deliver DNA into cells.⁷⁰ TAT peptide conjugated to cationic liposomes efficiently transduced cells in vitro and tumor *in vivo*.⁷¹ This could be effective strategy for therapeutic gene delivery. Furthermore, it was reported that TAT/PTD or Antennapedia complexed with recombinant retrovirus increased viral mediated gene delivery and protein expression in vitro and *in vivo*.⁷²

An efficient gene delivery agent with low cytotoxicity was generated by enzymatic digestion of the arginine-rich low molecular weight protamine (LMWP) with thermolysine.⁷³ LMWP showed similar intracellular localization and kinetics to those of TAT peptides and efficiently transduced and expressed the Lac Z gene. Compared to either, naked DNA or DNA/polyethyleneimine (PEI) complex, the LMWP/DNA showed significantly enhanced gene transfer while exhibiting markedly reduced cytoxicity.

A synthetically engineered PTD, named CFIS-R, was successfully designed to deliver DNA in vitro and *in vivo*.⁷⁴ CFIS-R consists of an arginine core sequence that facilitates DNA transduction. It is flanked with terminal cysteines to stabilize DNA condensation and histidine-glycine-histidine triplet interspersing every arginine residue completes the design. Spacing of the arginine core enhances gene delivery probably by protonation of the histidine groups thus allowing the plasmid to exit to the cytoplasm.⁷⁵ While six arginines are required for optimal in vitro transfection only three arginines are sufficient for *in vivo* gene transfer. The transfection is inhibited at 4°C which suggests that endocytosis is the mechanism of internalization with subsequent plasmid DNA endosomic escape. CFIS-R increases gene expression in both murine and human tissue *in vivo*.

17.4.2 DELIVERY OF OLIGONUCLEOTIDES AND PEPTIDE NUCLEIC ACIDS

The use of PTDs for delivery of oligonucleotides and peptide nucleic acid (PNA) has not been investigated as extensively as delivery of peptides and proteins. It is basically limited to TAT/PTD and Antennapedia delivery systems. Antisense oligonucleotides to the amyloid precursor proteins (APP) gene, successfully transfected cells and decreased the neosynthesis of APP expressed in glial and neural cells.⁷⁶ The change caused by the antisense oligonucleotide was transient but adequate to decrease axon and dendritic outgrowth in embryonic cortical neuron development. Enhancement of oligonucleotide delivery via TAT/PTD or Antennapedia conjugation, inhibited expression of Luciferase reporter gene.⁷⁷ TAT/PTD was utilized to deliver phosphorodiamidate morpholino oligomers (PMO) as well.⁷⁸ TAT-PMO conjugate targeted *c*-myc RNA and achieved nearly 100% inhibition of the expression at high doses, which lead to toxic side effects.

PNA is a molecule similar to DNA except an amino acid backbone replaces the sugar-phosphate present in DNA.⁷⁹ The amino acid backbone stabilizes PNA/DNA and PNA/RNA duplexes and protects them from degradation. PNAs have great potential for antisense applications because of their biological resistance to degradation and ability to bind complementary mRNA with stability and specificity. However, PNAs are not capable of crossing cell membranes. Several PNA molecules directed against oncogene PM1 RNA, or telomerase and galanin receptor RNAs have been described.^{80–82} PTD conjugated PNAs have been generated to study *in vivo* characterization and to measure biological processes at the cellular and molecular level (molecular imaging).⁸³ Intracellular MRI contrast agents were developed based on a PNA directed against oncogene c-myc conjugated to Antennapedia. An increased signal intensity in the tumor cells was observed in vitro and *in vivo*, using a *c*-myc-specific PNA compared to a non-specific PNA control.

A novel antibacterial therapy was developed based on PNA delivery by PTDs. The entry of foreign molecules in the bacteria is restricted by the lipopolysaccharide (LPS) layer and the inner and outer bacterial membranes. However, the delivery of a PNA against an essential fatty acid biosynthesis protein (acp) was achieved by conjugation to a peptide, KFFKFFKFFK, that was able to cross the bacterial membranes.^{84,85} Treatment with anti-acp PNA-KFFKFFKFFK completely suppressed bacterial growth. Moreover, cell cultures, noninvasively infected with *Escherichia coli* K12, were decontaminated by transduction with anti-acp PNA.

17.5 CONCLUDING REMARKS

The discovery of PTDs and their ability to translocate cellular membranes, with high efficiency and low toxicity, opened the possibility for intracellular delivery of proteins and peptides, DNA, antisense oligonucleotides, PNA and even nanoparticles in vitro and *in vivo*. Basic issues such as mechanism of cell

membrane translocation, immunogenicity and distribution within cells, have to be more deeply understood in order to have an optimal design of PTD molecules. Nevertheless, this technology already points out in the direction of a new and exciting way of generating novel molecules for research and therapeutic applications.

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18 Introduction to Therapeutic Nucleic Acids

Andreas G. Schätzlein

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18.1 INTRODUCTION

The sequencing of the human genome and that of increasing number of pathogens provides an unprecedented opportunity to harness the power of genetic therapies. The expression of exogenous foreign or homologous genes under the control of various promoters¹ has led to the development of various elegant therapeutic strategies in particular for the treatment of monogenetic disorders or cancer through the expression of proteins.^{2,3}

On the other hand increasing appreciation of the systems involved in the regulation of gene expression opens alternative avenues for the development of genetic therapies based on oligodeoxynucleotides (ODN). Oligonucleotides modulate gene expression at the level of translation, transcription, and in some cases at the epigenetic level using a number of mechanisms (Figure 18.1). This chapter will give an overview of the various strategies that have been developed to exploit this therapeutic potential.

Nucleic acids are special molecules as the same class of molecules has the potential to fulfill conceptually very different therapeutic functions:

- Informational storage and delivery of genetic code (e.g., genomic DNA, plasmids, gene repair)
- Three-dimensional structure enables specific binding to non-nucleic acid macromolecules (receptor/ligands)
- Functional/catalytic inhibition of cellular processes or cleavage of cellular nucleic acids (NA) through binding of the therapeutic NA (antisense, ribozymes, spliceosome).

This flexibility is linked to the ability of NA to (a) bind specifically to complementary NAs and (b) form secondary structures based on binding with in a single strand of NA.



FIGURE 18.1 Overview of cellular targets of the various therapeutic oligonucleotides (ODNs). ODNs modulate gene expression through up or down regulation of key steps at the various levels from transcription to translation. Detailed description of the particular mechanisms can be found in the respective text sections.

RNA as part of the cellular maintenance machinery and is represented in three main forms, the messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA). Other types of DNA exist in smaller quantities with various functions and new types are still being identified. Examples of less frequent RNA types are the small nuclear RNA (snRNA), small nucleolar RNA (snRNA) and the 4.5S signal recognition particle (SRP) RNA. RNA also plays a role as part of the telomerase enzyme which is involved in the maintenance of the telomere ends of chromosomes. Thus, RNA is the only biopolymer, which fulfills information storage (like DNA) and enzymatic functions.

18.2 NUCLEIC ACIDS

The natural nucleic acid molecules consist of a base, a sugar, and a phosphate residue. There are two chemically distinct types of nucleic



FIGURE 18.2 Nucleic acid chemical structure. Pyrimidine (uracil, thymine, cytosine) and purine (guanine, adenine) bases together with the ribose sugars and the phosphate form the nucleotides which form the basis of the nucleic acid polymers.

acids: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). They differ in the type of sugar and one of the bases.

The nucleic acid bases are heterocyclic compounds based on pyrimidine and purine, respectively (Figure 18.2). The pyrimidine bases are uracil (Ura), thymine (Thy), cytosine (Cyt) while adenine (Ade) and guanine (Gua) are purine based. In the natural nucleic acid polymers RNA contains AUGC whereas in DNA U is replaced by the 5-methyl derivative T to form the combination ATGC. Other highly modified bases occur naturally in the tRNA, e.g., pseudouridine, wybutosine, dihydrouridine, but also thymine (otherwise only in DNA) and methylated nucleotides. The respective nucleosides or deoxyribonucelosides (adenosine (A), guanine (G), uridine (U), thymidine (T), and cytidine (C)) are formed through N-glycosidic linkage between the C1 of a ribose or 2-deoxy ribose with the N1 (pyrimidine) or N9 (purine) of the nucleic acid base, with the sugars in furanose form. The esterification of the 5'-OH-group with phosphoric acid creates the phosphate derivatives or nucleotides. From the monophosphate derivatives, e.g., adenosine 5'-monophosphate (AMP), the di-, and triphosphates, e.g., ADP, ATP, are derived by addition of further phosphate residues through acid anhydride bonds.

Coupling of nucleotides by acid anhydride bonds is important for the creation of various co-enzymes (e.g., NAD⁺, NADP⁺, or coenzyme A) but is less useful for the assembly of larger chains. On the other hand, the reaction of the 5' phosphate of one nucleotide with the 3'-OH-group of a second nucleotide forms a nucleotide diester. This $5' \rightarrow 3'$ bond leaves the resulting dinucleotide with a free phosphate on the 5' and a free OH group on the 3' end of the chain ready for further chain extension. It is this straightforward reaction on which synthesis of oligonucleotides and polynucleotide chains and macromolecules is based and which forms part of the attractions of oligo- and polynucleotides. The ribose based ribonucleic acids (RNA) and the 2-deoxy ribose based deoxyribonucleic acids (DNA) are extended polynucleotides chains which for the ability to store genetic code are often called informational polymers.

18.2.1 POLYNUCLEOTIDE STRUCTURE

Formation of hydrogen bonds and ability to form secondary structures is critical for the therapeutic use of NA.

18.2.1.1 Double Stranded Nucleic Acids

DNA from all sources contains approximately equal amounts of A and T as well as G and C, but the total of both combinations will be different. This observation is explained by the model of Watson and Crick (1953) who postulated that DNA in fact consists of two strands linked through the H bonds between paired nucleotide bases (Figure 18.3). As suggested by the ratio of nucleotides the pairing is A–T and G–C according to their ability to form complementary base pairs. The hydrogen bonds are formed between the donors (amino and ring NH groups) and acceptors (carbonyl O, ring N) of a purine and a pyrimidine base, respectively, giving three H bonds between G-C and two H bonds between A-T.

The formation of H bonds is only possible if both strands run in opposite directions and complementary nucleotides and DNA sections are "teamed" up. The two strands are also intertwined to from a double helical structure with a pitch of 3.4 nm. In this typical B-DNA conformation the center of the helix is apolar, whereas the backbone with sugar and phosphate residues is negatively charged. Other less common DNA confirmations are the A and Z confirmation. In all cases the noncovalent interaction of the complementary



FIGURE 18.3 (See color insert following page 512) The structure of DNA. Hydrogen bonding between defined residues of a purine and pyrimidine based nucleotide (AT/GC) leads to Watson–Crick base pairing between homologous regions of the different strands which in the case of the deoxy ribonucleic acids (DNA) can form a double helical superstructure with a major and a minor groove.

strands can be disrupted and the strands separated reversibly by heat denaturation.

The noncovalent bonds are broken over a narrow temperature range of 85 to 95°C. The denaturation of chain melting can be monitored using the hypochromic effect: the stacked ring systems of the bases in the double helix absorb UV light to a lesser extent (\sim 40%) than that of the equivalent amounts of free bases. Loss of this structure, i.e., denaturation, can thus be measured as an increased UV absorption. The exact temperature at which this "chain melting" occurs depends on the exact composition of the NA. As G–C pairs contribute three and A–T only two hydrogen bonds the Tm increases about 0.4°C for each percent of additional G–C.

Re-naturation, the reversal of this process, occurs on slow cooling of the heat denaturized mixture. This allows the single strands to align so that complementary parts can start formation of double helical regions which then extend further from this starting point.

18.2.1.2 Single Stranded Nucleic Acids

The ability of RNA molecules to form complex secondary structures (Figure 18.4) is crucial for their biological role but has also been exploited when nucleic acids are used as nonhybridizing ligands in a general sense, i.e., for the binding to proteins and peptides (see below). In contrast to DNA, the steric hindrance through the hydroxy group at position 2' prevents the formation of an extended double helix in RNA. Consequently base pairing occurs over shorter distances and overall the three-dimensional structure of RNA is less stringent and more flexible than that of DNA. While the single-stranded NA polymer exists initially as a random coil it is possible that intra-strand hydrogen bonds can be formed between neighboring parts within the same chain if these contain complementary sequences. The chain folds back on itself to create a hairpin which consists of a loose loop (unpaired) followed by a helical "stem" (base-paired) in the region of complementarity (Figure 18.4). Other secondary structures which result from unpaired regions of DNA are



FIGURE 18.4 The structure of RNA. Single-stranded RNA can form extended superstructures. Secondary structures introduced through pairing between intra-strand homologous regions supports the formation of loops, bulges, and stems which form the basis of complex three dimensional structures but can also limit accessibility of mRNA for therapeutic ODNs.

interior loops and *bulge loops*. The bulge loop is formed to accommodate unpaired sequence in one part of the strand which is not present in the otherwise complementary region. The internal loop is a structure which is formed by corresponding sequences between otherwise complementary regions. RNA is the predominant single-stranded NA but principles are similar for single-stranded DNA.

Therapeutic oligonucleotides can be classed according to their main mechanism of action into antisense oligonucleotides (ASO), decoy ON, catalytic oligonucleotides/ribozymes, aptamers, triplex forming oligonucleotides (TFO), or RNA interference based (Figure 18.1). These technologies have reached various stages of maturity ranging from exploratory research to advanced clinical development. Antisense based therapies probably most advanced in their clinical development and second- and third-generation antisense oligonucleotides are under development. The numerous chemical modifications which have been employed in an effort to improve nuclease resistance, binding affinity or pharmaceutical properties will be covered in some detail in conjunction with the antisense oligonucleotides, but are in principle applicable to most other types of ODNs.

18.3 ANTISENSE OLIGONUCLEOTIDES

Antisense oligonucleotides (ASO) are short (13–25 nt) single-stranded nucleic acids which disrupt expression of a target protein by sequence specific binding to the complementary mRNA. Since its discovery in 1978, antisense strategies for the specific reduction of expression of a number of therapeutically relevant genes have been in development and are now in clinical trials.^{4,5}

The principle of antisense is relatively straightforward: Small (13–25 nt) single stranded ON bind to RNA in a sequence-specific manner through Watson–Crick base pairing. Knowledge of the target gene sequence should in principle be sufficient for the design of the ASO. The resulting ASO/RNA complex leads to a downstream reduction in the expression of the encoded gene via a number of potential mechanisms which involve inhibition of splicing, mRNA degradation, and inhibition of translation. The degradation of target mRNA through ASO guided RNase H catalyzed cleavage is probably the most important route.

For the clinical application of ASO the stability in serum (nucleases) and the specificity of the knock-down effect have been critical challenges that are being addressed through chemical modification of the NA backbone or the use of delivery systems.

18.3.1 MECHANISMS

18.3.1.1 RNase H Mediated mRNA Degradation

RNase H dependent cleavage of mRNA is the most effective and frequently used mechanism for antisense knockdown.⁶ This nuclease is present in the

nucleus of most cells and reliably cleaves complexes which span the specific RNase H cleavage sites. RNase H cleavage can reduce the level of target RNA by 80 to 85%.⁷ RNase H cleavage sites are mainly found near the translation initiation codon and the 3' and 5' untranslated regions, the coding regions seem to be less suitable. A 5 bp homology region sufficient in vitro to achieve a down regulation of gene expression but specificity may not be sufficient; the Isis 3521 22mer targeting PKC-alpha also downregulates PKC-zeta with 11 bp homology.⁸

Not all chemically-modified ODNs are permissive for RNase H cleavage, but it is the main sequence specific mode of action for the unmodified phosphodiester backbone and the first-generation phosphorothioate backbone antisense oligonucleotides (PSO). The advantages of PSO chemistry which do not normally support RNase H mediated cleavage such as, e.g., 2'-modified RNA, morpholino derivatives or locked nucleic acids (LNAs) (see below) can be combined with standard phosphodiester linked DNA in chimeric molecules: if the standard DNA is included as a central stretch of at least 5 nt these so-called "gapmers" become amenable to RNase H cleavage.^{9,10}

18.3.1.2 Steric Hindrance of Translation

ASO which do not activate RNase H but hybridize with the target mRNA can sterically inhibit translation. The exact mode of action depends on the region of the mRNA the antisense molecule is targeted to: hybridization to the 5' UTR terminus or AUG initiation codon can block ribosome binding whereas those targeting regions downstream of the initiation codon act as "roadblocks" which may prevent elongation. This mechanism has been demonstrated by the presence of abortive peptides after PNAs and morpholino backbones hybridization to mRNA.

18.3.1.3 Antisense Modulation of mRNA Splicing

An estimated 60% of the 30,000 human genes undergo alternative splicing creating gene variants with modified functionality.¹¹ Errors in the splicing process introduced through mutations are involved in about 50% of genetic disorders such as β -thalassaemia, cystic fibrosis, Duchenne muscular dystrophy, and cancer. In contrast to the interaction of antisense molecules with the mRNA which leads to reduced gene expression through message degradation and inhibition of translation the interaction of ASO with RNA before the splicing can change the ratio of various splicing variants, block the processing of erroneous sequence and promote splicing of repaired variants.¹¹

Chemically the ODNs used for modulation of gene splicing need to come from derivatives which do not activate RNase H, as this would lead to mRNA degradation. Overall affinity to target sequences must be high enough to compete with the nuclear splicing factors for binding to pre-mRNA. So far it is predominantly oligonucleotides with modifications at the 2' position (2'-Omethyl/methoxyethyl/aminopropyl) and morpholino derivatives which have been used but other chemistries such as PNA also have similar properties. Recently a study reported the use of 2'-O-methoxethylene modified oligonucleotides to redirect polyadenylation of the pre-mRNA away from one of the alternative polyadenylation sites,¹⁴ thus potentially modulating stability of the transcripts.

Examples of successful use of this strategy are the repair of the thalassemic human beta-globin mRNA¹² and the re-introduction of the apoptotic effects of Bcl-xS by balancing of the Bcl-x splice variant ratio,¹³ but numerous others are being explored.¹¹

18.3.1.4 Nonsequence Directed Activity

The effects of antisense oligonucleotides which are not related to their binding to a target sequence are difficult to predict and control. Immune stimulatory effects have been observed in conjunction with specific backbone chemistry as well as the use of specific bases: strong immune mobilising effects can be induced through unmethylated CG nucleotides in a specific sequence context, the so-called CpG motif (–GTCGTT–).¹⁵ This response forms part of the immune system with the specific task of recognizing foreign, in particular microbial, DNA which in contrast to vertebrate DNA does not carry the methylated CGs. Certain immune cells, e.g., dendritic cells, have evolved the capability to recognize the lack of methylation within the specific context and subsequently trigger an immune response.^{16,17}

The polyanionic nature of the phosphorothioate backbone has been linked to effects such as protein binding, prolongation of partial prothrombin time and complement activation independent of the sequence of specific nucleotides. PSO can also induce cytokines (e.g., IL-6, IL-12, TNF- α , IFN- γ , IL-1) and chemokines (reviewed in Ref. 18). The profile of nonsequence specific AS ODN effects is likely to be skewed against phosphorothioate backbone ASO because of the fact that the clinical development of those materials (first generation) is most advanced and the nonantisense effects well understood.

The nonspecific effects of ASO are in general not desirable because they can interfere with the specific action of the ASO and potentially trigger side effects. However, when properly harnessed the immune stimulation through antisense clearly has therapeutic potential and can in fact be regarded as an additional nonsequence specific mode of action of ASN. The therapeutic potential of this approach is currently being explored in trials with cancer patients.^{19,20}

18.3.2 CHEMISTRY

A variety of chemical modifications have been developed in order to overcome some of the limitations of the natural phosphodiester backbone¹⁸ (Figure 18.5). Changes to the NA backbone are particularly required in order to enhance the stability of ASO to the attack of ubiquitous nucleases, improve general uptake and bioavailability, and increase strength of the specific interaction with target mRNA. The breakdown products of this degradation, dNMP



FIGURE 18.5 Chemical modification of ODNs. Examples of chemical modifications which have been introduced in an effort to increase the nuclease resistance of nucleic acids. The natural phosphodiester backbone is depicted schematically in the center. The first generation of modifications focused on the replacement of an oxygen in the backbone. The use of sulfur in this position leads to the phosphorothioate ODNs. Further modifications include the phosphoramidate ODN (PSO) and peptide nucleic acids (PNAs).

mononucleotides, have been shown to have nonspecific cytotoxic and anti-proliferative effects.

18.3.2.1 Methylphosphonates

Chemical modifications have been introduced to improve stability and specificity of ASOs. The first modification was the introduction of methylphosphonates in which nonbridging oxygen is replaced by a methyl group (Figure 18.5). While this modification renders the ODN very stable it also reduces its charge, which in turn reduces cellular uptake and solubility. Furthermore, methylphosphonates lack the ability to activate RNase H.

18.3.2.2 Phosphorothioates

The replacement of the nonbridging oxygen by sulfur results in a phosphorothioate (PS) backbone (Figure 18.5). PSOs represent the majority

of ASO currently in clinical development and the first licensed drug, Vitravene, is also based on this chemistry.

The modification introduces chirality and results in a relatively nuclease resistant Sp and a less stable Rp diastereomer. The PSOs have good antisense activity, are water-soluble and able to activate RNase H, properties which have made this probably the most frequently used modification.²¹

One of the major issues that have been linked to the use of the PS backbone is its nonspecific, length dependent interaction with various cellular proteins, specifically a number of growth factors. While this nonspecific interaction may be difficult to control it has been suggested that on the clinical level the interaction with a range of growth factors may actually form a substantial contribution to the clinical responses that have been observed.²¹ The PSOs are polyanions with poor cellular uptake and reduced complex stability compared to unmodified ON (~0.5°C per nt; reviewed in Ref. 18). In vivo they show favorable pharmacokinetics, probably related to their binding to proteins, and uptake in most organs, in particular liver, spleen, kidney, but also in tumors.²²

18.3.2.3 Morpholino Oligomers

The morpholino Oligomers are nonionic DNA analogues with substantially altered chemistry (Figure 18.5). Morpholino-Oligomer/RNA duplex formation is facilitated by the uncharged backbone but the overall affinity is similar to that of unmodified ODNs. The lack of charge is potentially advantageous in avoiding nonspecific effects related to the strong protein binding. Morpholino Oligomers have been used successfully to modulate gene expression during development (various articles²³) and it has been suggested that their backbone may facilitate binding to mRNA with secondary structure by strand invasion or that the RNA–ON duplex is more disruptive during translation.²⁴

18.3.2.4 Peptide Nucleic Acids

Peptide nucleic acids (PNA) are DNA/RNA mimics based on *N*-(2aminoethyl) glycine backbone and methylene carbonyl linked nucleobases.^{25,26} Complementary strands are recognized by Watson–Crick base pairing and binding occurs with improved affinity. PNA are not substrate for either proteases or nucleases and the neutral backbone reduces protein binding. Synthesis can be carried out by standard peptide chemistry methodology with facile attachment of further peptide based functional groups (e.g., membrane penetrating). The PNA antisense mechanism is based on steric inhibition of translation rather than enzymatic degradation as PNA are not substrate of RNase H.²⁷

In addition to their ability to act as antisense ODN PNA have the ability to interact with double stranded DNA by strand invasion and formation of quadruplex in which one PNA strand binds through Watson–Crick interaction and a second by Hoogsteen base pairing.²⁸ This makes PNA chemistry very interesting for the use in triplex forming oligonucleotides (TFOs, see below).

18.3.2.5 Locked Nucleic Acids

Locked nucleic acids (LNA) (also bridged NA, BNA) are analogues of RNA and DNA which are constrained through a methylene link between ribose 2'-oxygen and the 4'-carbon. The linkage can be through oxygen, sulfur, or amine and leads to significantly improved binding to complementary DNA and RNA sequences. Depending on the specific base, melting temperature (T_m) is increased by several degrees (3 to 8°C) for each LNA monomer leading to high affinity hybridization. The modification also makes LNA-containing ODNs more stable in serum. While LNA do not activate RNase H it is easy to synthesize chimeric LNA-DNA "gapmers" containing a central DNA stretch of 7 to 8 monomers which combine stability with RNase H activation. In vivo the LNAs seem to be well tolerated and have been shown to be effective in inhibiting tumor growth in a murine xenograft model. LNAs have also been used to add functionality to double-stranded DNA in plasmids apparently hybridizing after strand displacement similar to PNAs.²⁹

18.3.3 APPLICATIONS

With the recent approval of the first antisense drug, Vitravene (Formivirsen) against cytomegalovirus retinitis in AIDS patients, by the Food and Drug Administration (FDA) antisense therapy has come of age.^{5,30} Late phase clinical trials are in progress against a number of antisense targets, particular in cancer.^{15,31} A recently updated review³² lists 35 clinical trials with antisense compounds as of the beginning of 2003. The target indications range from genital warts (Mircologix MBI1121) to cancer (c-myc, VEGFR1, HER2) and HIV. Most of the compounds are based on first-generation chemistry, i.e., PS ODNs. It has been suggested that the nonspecific effects of AS ODNs, in particular of PS ODNs, may contribute to any observed effect. In many clinical situations, such as cancer effects, immune stimulation may however be actually beneficial and some oligonucleotides exploit this effect directly.^{19,20}

Despite the advanced clinical development of ASO there remain a number of questions outstanding. One intriguing observation that has been made with various oligonucleotides is that in tissue culture the compounds tend not to gain entry into the cells without the use of delivery systems.^{33,34} Among the systems in development are synthetic systems as they are familiar from the delivery of plasmid DNA such as cationic lipids and polymers but also oligonucleotides linked to cell penetrating peptides.³⁵ The efficiency in animal models and clinical trials has been demonstrated after systemic administration of the oligonucleotides alone and recently oral bioavailability of secondgeneration oligonucleotides was demonstrated.^{36,37} The question of the contribution of non antisense mechanism contributing to biological effects in vivo and the specificity of antisense for the various backbones are also not fully understood. It has been estimated that for PSO RNase H mediated knockdown of gene expression can reach levels of 80 to 85%.⁷ There is evidence of additive effects of combined use of ASOs and siRNA. An estimation of relative efficiency of AS ODN in comparison to siRNA based gene knock-down is interesting but inherently difficult. A study comparing the effects of P2X3 knock-down found siRNA requiring only 1/10th of the dose of the ASOs but both probes demonstrating an additive effect when given in combination.^{38,39}

18.4 DECOY NUCLEIC ACIDS

Double-stranded DNA sequences which interact with proteins based on sequence-defined Watson–Crick base pairing and thus sequester the protein away from its natural interaction partner. In general the NA binding proteins have a role as regulators or gene expression, i.e., modulators of transcription such as transcription factors (Figure 18.6). Single stranded NA can also act as decoys for specific proteins such as those which recognize viral RNA. Theoretically decoys could also bind to proteins as aptamers (nonbase pairing), i.e., the interaction is based predominantly on molecular shape (i.e., secondary structure) rather than a specific sequence.

18.4.1 MECHANISM

Double-stranded NA sequence normally required for binding of transcription factors can be mimicked by palindromic single-stranded hairpin ON which self-hybridizes and thus is able to fold back on itself.^{40,41}



FIGURE 18.6 Model of decoy ODN activities. Decoy ODNs interact with proteins such as transcription factors (TF) in a sequence specific fashion. Decoy ODNs binding to a transcription factor compete with the natural ligands; this sequestration of transcription factor leads to a reduction of gene expression. Alternatively decoy ODN could inhibit repressors to increase expression.

Disadvantage of conventional phosphodiester ODN is the rapid degeneration by cellular nucleases. In an effort to overcome this limitations chemical modifications such as the use of phosphorothioate and methylphosphonates were introduced but often led to other problems such as immune activation, loss of specificity, and RNase H sensitivity.⁴² Recently so-called circular dumbbell double-stranded decoy ODN were created by ligation of two identical double stranded ODN which have been reported to be more nuclease resistant and have improved transport characteristics.⁴²

The potential disadvantage of the transient nature of the binding based on base-pairing has been addressed recently though chemical modification.⁴³ A chemically-modified hairpin ODN carrying an integrated diphosphoryldisulfide linkage bound covalently to the transcription factor NF κ B and thus acted as decoy for the natural substrate.

While the binding of transcription factors in general requires doublestranded DNA for sequence-specific binding, decoy strategies can also be based on single-stranded DNA if the interacting protein recognizes specific sequences such as viral RNA sequences employed to bind and recruit selectively proteins required for replication. Decoy RNA concepts demonstrated using HIV RRE and TAR sequences. The TAR sequence recruits TAT protein and decoy RNA competes and sequesters TAT protein reduces viral transcription and expression of the decoy in CD4+ cells inhibits HIV replication and gives these cells a survival advantage. A similar strategy based on RRE has been evaluated in the clinic.⁴⁴

18.4.2 APPLICATIONS

The therapeutic potential of decoy ODN has been demonstrated in a number of animal models of, e.g., glomerulonephritis, rheumatoid arthritis,⁴² and aortic aneurysm.⁴⁵ A decoy to the transcription factor E2F is under clinical development for the prevention of restenosis after venous or coronary vascular grafts.^{46,47} The safety of the Rev RRE decoy as inhibitor of HIV-1 replication has recently been demonstrated in haematopoietic stem cells.⁴⁴

Another example is the targeting of ODN against telomerase, a ribonucleoprotein which contains a RNA domain. Telomerase is involved in the maintenance of telomere on the end of chromosomes during cell division. Normally cell proliferation is limited when telomeres have shortened beyond a critical length. Telomerase which is not active in normal tissue but frequently in cancer cells maintains telomerase length and thus supports further proliferation. ODN targeted against the RNA domain of telomerase act as decoy for the natural substrate and have been shown to inhibit proliferation.⁴⁸

18.5 CATALYTIC NUCLEIC ACIDS

The discovery of catalytically active RNA in the 1980s lead to a paradigm shift away from the view of proteins based enzymes as the only catalytic biological molecules. Since then the important role of catalytic RNA in the synthesis of RNA components has increasingly been recognized.

The reactions catalyzed by these so-called "ribozymes" NA include quasicatalytic reactions in which intramolecular reactions leave the molecule modified with a single turnover but can also facilitate *trans* reactions with high turnover and no modification.⁴⁹ Natural ribozymes have the ability to form and dissolve covalent bonds by transesterification, hydrolysis, and peptidyl transfer⁵⁰ but with the exception of RNase P only catalyse intramolecular reactions.

Substrate recognition is based on highly specific RNA–RNA interactions between a template sequence and the substrate. In larger ribozymes flanking sequences and tertiary structures guide and stabilize the substrate with respect to the catalytic site. In general the ribozyme sequences responsible for substrate recognition are well separated from the catalytic centers of the ribozyme. This facilitates the engineering of artificial ribozymes with modified sequence recognition sites and the ability for intermolecular cleavage.

Natural ribozymes can be put into three distinct classes: the self-splicing introns (group I and II), RNase P, and the small catalytic ribozymes which include the hammer head, hairpin, hepatitis delta virus (HDV), and the VS ribozymes.^{50–52} The smallest natural ribozymes (\sim 40 nt) is the extremely efficient self-cleaving hammerhead which mediates rolling-cycle replication in circular virus-like RNA in plants and which may have evolved multiple times during evolution⁵³). The hammerhead and hairpin ribozymes were subsequently the first to be used as prototypes for the design of trans-cleaving therapeutic ribozymes (Figure 18.7).

18.5.1 MECHANISMS

18.5.1.1 Trans-Cleaving Ribozymes

Trans-cleaving ribozyme strategies aim at reducing the load of harmful RNA, e.g., form virus infection by cleaving of the specifically recognized RNA. RNAcleaving ribozymes bind to the target RNA via Watson–Crick base pairing, depending on the specific ribozymes flanking sequences and tertiary structures stabilize the binding and assure proper orientation. The cleavage mechanism itself depends upon the architecture of the individual ribozyme. Frequently this involves destabilization of the backbone through a nucleophilic attack at the 2'-OH 5' from the cleavable bond. Once cleaved the fragments dissociate and release the ribozyme; the fragments are rendered untranslatable and will be further degraded. The efficiency of a particular ribozyme is largely dependent on the rate of binding, cleavage, and release.

One of the limitations of RNA based ribozymes is their sensitivity to degradation; on the other hand this makes them also amenable to vector based intracellular production. In fact, a vector based ribozyme strategy has been used to express multiple ribozymes against HIV from a single plasmid in a "shot-gun" approach.⁵⁴



FIGURE 18.7 (See color insert) Structure of a hammerhead ribozyme. A threedimensional model of hammerhead ribozyme structure based on fluorescence measurements; the RNA chain were rendered as arrows with base-specific coloured ladder (MMDB 1RMN1^{123,124}).

In the case of the hammerhead ribozyme the typical target site has a length of about 15 nt with 6 to 7 nt on either of the binding arms interrupted by the cleavage site. This conformation leads to a relatively weaker binding than a single 14 nt biding sequence but also makes the ribozyme more sensitive to even single nt variations in the target molecule and thus promotes high specificity of cleavage.⁵⁵

18.5.1.2 Trans-Splicing Ribozymes

An alternative to the enzymatic degradation of mRNA through the *trans*cleaving ribozymes is based on group I ribozymes. Group I and II introns are found in the genes of a number of lower eukaryotes and prokaryotes and differ from spliceosomal introns because of their ability to self-splice independently from the precursor RNA. If harnessed appropriately this ability may prove useful in potential gene revision/modification approaches.⁵⁶ In particular it is possible to design group I ribozymes that can correct transcripts by cutting upstream of mutation and splicing in of a corrected version of the transcript.⁵⁷ The ribozyme provides an exon-like replacement attached to its 3' end which can be spliced in immediately 3' to the cleavage site. The strategy can also be used to create chimeric proteins or to modify harmful proteins into harmless variants.⁵⁸

18.5.1.3 Emerging Strategies

There is evidence to support a more central view of RNA based enzymes during evolution,⁵⁹ but for many of the cellular roles of RNA molecules proteins take on a crucial supporting function. Some recent approaches exploit the fact that proteins can act as chaperons for ribozymes, guide and prepare the RNA substrate, or assure co-localization of the ribozyme and target.⁵¹ Chimeric RNA molecules can combine a catalytic domain with a decoy domain which recruits proteins to improve ribozyme performance. The combination of a hammerhead catalytic domain and a decoy domain which recruits helicase A and eIF4A proteins with helicase activity to increase accessibility of the substrate provides an example of this approach.⁶⁰

The regulation of the activity of ribozymes through external factors has been achieved using allosteric control through a number of small molecules (ATP, flavin mononucleotide, theophylline, cNMPs) or various oligonucleotides (reviewed in Ref. 51). Depending on the specific ribozyme construct activity would be increased for example in the presence of an external oligonucleotide which competes for binding with an intramolecular attenuator domain that initially blocks the active site. Alternatively the external oligonucleotide would complement a ribozyme so as to complete the substrate-binding domain.

18.5.2 MODIFICATIONS

Synthetic ribozymes need to be made nuclease resistant to withstand rapid degradation in nuclease-rich biological fluids.⁵⁵ Nuclease-resistant ribozymes have been constructed which contain chemical modifications predominantly at the 2'-OH position at each nucleotide.^{55,61} Chemical modifications include change of the 2'-OH position to the naturally occurring but more resistant 2'-O-Me for most of the ribozyme. Inverted deoxybasic sugar residues at the 3' end of the chain and up to four phosphorothioate groups at the 5' end are additional features which can help to stabilize the ribozyme without compromising its specificity as is sometimes observed with higher fractions of phosphorothioate in the NA backbone.⁵⁵ While a pure RNA hammerhead ribozyme was found to have a serum half-life of only a few seconds, the corresponding hybrid RNA–DNA ribozyme chimera half-life was extended to at least 4 min.

18.5.3 DNAZYMES

DNAzymes are a synthetic class of catalytic nucleic acids based on DNA backbones (reviewed in Ref. 62), where DNA and particular PS based molecules are resistant to nuclease degradation but do not have any endogenous catalytic activity. Based on an in vitro selection procedure enzymatically active sequences were identified which were able to cleave an RNA substrate specifically after binding to a recognition domain in the hybridizing arms.⁶³

18.5.4 APPLICATION

Small ribozymes such as hammerhead and hairpin ribozymes have provided the blueprint for the *trans*-cleaving ribozyme therapeutics with the clinical development focusing on viral diseases. Hepatitis B virus infection has been targeted via the HBx mRNA which codes for a transcriptional activator protein as well as targeting of the polymerase and X protein.^{64,65} In hepatitis C with the viral genome present as RNA hammerhead ribozymes targeted to the 5' UTR region significantly reduced mRNA load and led to infection resistance and a trial using a modified ribozyme ("Heptazyme") is in clinical development.⁵⁵ Another target for the *trans*-cleaving ribozymes are various HIV RNA such as *tat*, *env*, *gag*, or *pol*. Lymphocytes and haematopoietic precursors were transduced with an anti-HIV hammerhead expressing retroviral vector ex vivo and the re-infused and transduced cells were found to persist in patients for up to a year.⁶⁶ Other targets in clinical development target cancer related genes such as MDR-1 and flt-1 (VEGF high affinity receptor) and HER2.⁵⁵

The clinical development of ribozyme therapies is farthest advanced with the *trans*-cleaving synthetic nuclease resistant ribozymes "Angiozyme," "Heptazyme," and "Herzyme" as well as a retroviral construct against HIV tat and rev exons are in clinical trial.^{55,67}

18.6 APTAMERS

Aptamers are single-stranded nucleic acids which can form complex threedimensional structures (Figure 18.8).^{68,69} Specific aptamers can be made which will bind with high affinity and specificity to other molecules based on their sequence-specific structure but not sequence directed fashion, i.e., Watson– Crick interactions.⁷⁰ Therefore, aptamers can also efficiently bind to proteins and other nucleic acid macromolecules. One important advantage of aptamer technology is that high affinity aptamers can be identified iteratively using powerful in vitro selection techniques (Figure 18.9). The so-called systematic evolution of ligand by exponential enrichment (SELEX) and related techniques allow rapid selection of aptamer sequences which bind to virtually any given protein.⁶⁹

In vitro selection strategies for the identification of suitable NA sequences as ligands to protein targets have been developed by several groups and are similar to the SELEX procedure proposed in Refs 70 and 71 (Figure 18.8).

A combinatorial library of single-stranded NA is created by chemical synthesis containing 10^{14} – 10^{15} different oligonucleotides of a typical length of 20–40 nt.¹¹ The library is then incubated with the purified target protein under suitable conditions to allow binding of sequences which form high affinity structures. Unbound sequences are then separated (e.g., by elution) and discarded. The high affinity binders are recovered from the protein and undergo a suitable amplification before the process is being repeated. This procedure leads to an enrichment of the high affinity binders in the selected library. With each round of selection and amplification (typically around 8–12)

FIGURE 18.8 (See color insert) Aptamer 3D structure. A theophylline-binding aptamer in complex with theophylline; the theophylline was rendered as standard CPK, the RNA as arrow with base-specific ladder (MMDB 1015¹²⁴).

the binding and elution conditions become more and more stringent so that the fraction of high-affinity binding sequences increases exponentially. The specific amplification procedure will depend on the chemical nature of the NA: for DNA based libraries PCR reactions are used, in the case of RNA based libraries it is a combination of reverse transcription and PCR. After the final round the sequence(s) of the selected library is determined by sequencing.

The procedure leads to the selection of very high affinity binders with dissociation constants typically in the low nanomolar to picomolar range and can discriminate between targets that are 96% identical. In their binding characteristics these aptamers are also in general very specific and thus similar to antibodies or Fab fragments.⁷⁰ However, it is crucial that the conditions chosen during selection appropriately mimic the conditions likely to be found during the later application. This includes factors such as temperature, cation concentrations, pH which will influence the conformation of the aptamer or the target protein. On the other hand controlled changes in these conditions can be used conveniently to elute and recover aptamer after a binding event.

18.6.1 MECHANISM

Aptamers functionality is broadly in parallel with antibodies and antibody fragments or phage peptide display which can be seen as a directly competing technology. One particular advantage of aptamers over the competing antibody technologies is the broad range of suitable target which do not have to be immunogenic and can also be highly toxic.⁷² In fact for reasons which are still not fully understood synthetic aptamers seem to be virtually nonimmunogenic even in experiments designed to provoke an immune response.⁷⁰ Aptamers also have potential advantages related to their size (smaller then single-chain Fab fragment but frequently with higher affinity) and nonproteinaceous



FIGURE 18.9 Aptamer selection by systematic evolution of ligand by exponential enrichment (SELEX). A library of ONS (1) and the molecule for which an aptamer is to be selected (2) and incubated under conditions which allow binding of aptamers with a suitable structure (3). Various techniques are used for the separation (4) of unbound ODNs (5) and bound aptamers (6). Under more stringent conditions the binding molecule (7) and the aptamer ODNs (8) are separated and the selected aptamers population is then amplified (9) to enter into further rounds of selection.

nature and have been likened more to complex chemicals as opposed to biologicals.⁷³

Aptamers when selected and optimized appropriately can be highly specific and distinguish between various protein isomers and conformations as well as between phosphorylated and unphosphorylated forms of proteins. Interestingly, this specificity is not achieved by one specific mechanism of binding but in facts reflects the flexibility of interactions between aptamers and the target molecules.⁷²

18.6.2 CHEMISTRY

RNA, DNA, and modified RNA are used to produce aptamers. In order to transform the in vitro selected NA sequence into a potential therapeutic agent

suitable for *in vivo* administration several optimization strategies are employed. Overall this optimization is largely empirical and requires frequent re-testing of the aptamer. Specific aspects that need to be optimized to address biological issues include aptamer length and backbone chemistry.

In order to make cost efficient synthesis possible aptamer size needs to be minimized so as to obtain active binders with less than 40 nt. Very small aptamers on the other hand are easily eliminated via filtration in the kidneys and thus have a shorter plasma half-life.

Another important aspect of *in vivo* activity is the stability of the resulting aptamer in biological fluids, specifically in the bloodstream which depends mostly on its backbone chemistry. While RNA backbones can be degraded in plasma within a few seconds DNA has been found to be significantly more stable (30 to 60 min). Chemical modifications, e.g., capping of 3' ends or substitution of ribonucleotides with amino, fluoro- and alkyl derivatives which are amenable to inclusion into the SELEX process, can result in aptamers which are stable in plasma over several hours.

18.6.3 SPIEGELMER

The expression "Spiegelmer" (spiegel = German for mirror) has been coined to describe mirror images of the more conventional aptamers based on L-nucleic acids.⁷⁴ As these forms of nucleic acid (L-DNA, L-RNA) are unknown in nature no enzymes exist for their degradation thus removing the need for stability driven changes to the backbone. The selection utilizes the principle of chiral inversion: an aptamer which binds to the synthetic enantiomer of the target molecule (Based on D-amino acids instead of the normal L-amino acids) when synthesized as L-enantiomer (Spiegelmer) will bind to the original L-amino acid based peptide.⁷⁵ Spiegelmer binding to the various biological peptides have been identified, e.g., vasopressin⁷⁶ or GnRH.⁷⁷

18.6.4 APPLICATIONS

Aptamers have proven interesting in a variety of interesting areas such as tools for the elucidation of signal transduction pathways, antibiotics research, diagnostic tools, or biosensors.⁷² For example, aptamers against specific intracellular targets, so-called "intramers," can be produced in the cell by various expression systems and have potential utility in functional proteomics and high-throughput target validation efforts.⁷⁸

A similar system for the intracellular expression of aptamers also has potential therapeutic implication, e.g., to suppress the expression of oncogenes or growth factors such as NF κ B where the aptamer acts as a decoy.⁷⁹ While this aptamer has been selected based on its structure related binding properties decoy NA against transcription factors tend to be designed based on their natural NA interactions.

Another interesting development are ligand-regulated aptamers which allow gene-specific small ligand controlled translation. Ligand specific aptamers sequences at the untranslated 5' end of genes prevents expression of specific genes in the presence of the small ligand/drug which binds to the aptamer region and thus blocks translation.⁸⁰ A similar mechanism seems to be involved in natural regulation of metabolic processes in bacteria.⁸¹

The diagnostic use of aptamers as the target specific element, e.g., in radiological probes for *in vivo* imaging of cancer related epitope has recently been reviewed.⁸² Based on the pharmacodynamic and pharmacokinetic properties of aptamers it has been suggested that they would be most suited to the treatment of conditions which are acute by nature and related to important spatially confined extracellular targets where no satisfactory alternative forms of treatment currently exist.⁷⁰ Vascular targets such as those linked to coagulation and thrombosis, intima hyperplasia and angiogenesis fulfil these criteria and therapeutic aptamers are actively being developed for related conditions. The development of the aptamer NX 1838 against vascular endothelial growth factor (VEGF) has progressed quite far. In its PEGylated form ("Macugen," pegaptanib sodium) the compound has entered into phase III clinical trials exploring its efficiency against age-related macular degeneration⁸³ but is also being explored in a number of other disease models.⁷²

18.7 TRIPLEX FORMING OLIGONUCLEOTIDES

The phenomenon of triplex formation between the DNA double helix and a third strand was first reported in 1957.⁸⁴ While binding of the third strand was comparatively weaker it was found to be sequence specific and found to be able to inhibit transcription.⁸⁵ Triplex forming oligonucleotides (TFO) have potential to interact with genomic DNA and messenger RNA to modulate gene expression in a therapeutic fashion (Figure 18.10). TFO bind to the major groove of double-stranded DNA using Hoogsteen bonds rather than the Watson-Crick bonds that are established between the two anti-parallel strands of the DNA (Figure 18.10). The ability to bind to double stranded DNA in a sequence specific manner makes TFO potentially interesting tools for a number of therapeutic anti-gene and gene modification strategies.

18.7.1 TRIPLEX FORMATION

The formation of the Hoogsteen bonds places a number of structural contains on the TFO including the necessity for 15 to 30 nt stretches of homo-purine and homo-pyrimidine as binding site.⁸⁶ Stable bonds can be utilized through binding of the third strand in the parallel formation (pyrimidine motif) through the formation of the C⁺·GC triplets and the T·AT base triplets, or through the purine motif which runs anti-parallel in the groove using G·GC, A·AT, and T·AT Hoogsteen bonds.^{87,88} Both binding motifs have further limitations such as the dependence on low pH (C⁺·GC) or in the case of purine motifs the effects of ions which can inhibit (K⁺) or support (Mg⁺) triplex formation. Other factors known to influence the binding are the presence of secondary



FIGURE 18.10 DNA modifying activity of triplex forming ONs (TFO). TFO can interact with double-stranded DNA and can thus act at the level of transcription. Triplex formation in the promoter region of a gene can modulate binding of transcription factors (or repressors) by blocking of the natural interaction site or through recruitment via TFO tethered binding factors. TFO binding downstream of the promoter can inhibit transcription through steric hindrance (top). TFOs can act as site specific anchor which block transcription but can also direct tethered reactive ligands to specific sequences (middle). When short homologous ODNs are tethered to the TFO specific DNA regions can be repaired and recombination events triggered (bottom).

structures in the TFO (e.g., G tetrads), general accessibility of the target, and overall TFO affinity. The values for the TFO binding affinity constants should be better then $\sim 10^{-9}$ mol in order to achieve functional binding under biological conditions.⁸⁹

A number of chemical modifications (reviewed extensively in Ref. 88) have been introduced with a view to increase stability of binding and accelerate the rate of triplex formation; the modifications include the use of conformationally constrained NA, morpholino derivatives, and PNAs. The lack of phosphodiester bonds in the PNAs leads to a reduced charges repulsion from the backbone. In contrast to TFOs the PNAs displace the second strand of a duplex DNA and bind to the complementary strand or any single-stranded RNA via Watson–Crick interactions (strand invasion and P-loop formation). Triplex formation with a second strand of TFO occurs in the major groove of the PNA–DNA hybrid via Hoogsteen binding.²⁸ Recently it has been shown that conformationally constrained LNAs can also bind to double stranded pDNA in a mechanism similar to that of PNA.²⁹

18.7.2 THERAPEUTIC STRATEGIES

The ability of TFO to bind double stranded DNA makes TFO potentially interesting for a number of unique therapeutic approaches at the gene level.^{86,89}

18.7.2.1 Transcriptional Regulation

Binding of the TFO to the genomic DNA potentially limits access of other molecules. In the case of regulatory/promoter regions this can be used to inhibit the binding of transcription factors to prevent initiation of transcription. Binding of TFO downstream from the promoter will interfere with transcript elongation and thus block or reduce gene expression. Binding of TFO can potentially also upregulate transcription by inhibiting the binding of repressors⁹⁰ or through recruitment of transcription factors to the promoter region. It has been suggested that P-loops of the coding strand that are induced by the binding of PNAs resembles the structure of transcription bubbles and thus are casual in the increased transcription observed at nearby sites.²⁵ The transcriptional activation domains derived from herpes simplex virus protein 16 (VP16) were linked to TFO in a peptide–TFO chimera which increased transcription of a transgene from the linked plasmid.⁹¹

18.7.2.2 Genome Modification

The site specific binding of TFO to genomic DNA also offers the ability to introduce permanent changes to the DNA sequence by using triplex directed mutagenesis and triplex induced recombination events.

In the case of triplex-directed mutagenesis the TFO acts as a targeting ligand which directs the mutagenic agent, e.g., covalently linked psoralen, to the specific sequence. Interestingly the presence of a mutagen is absolutely required as the processing of the triplex through the nucleotide excision repair pathway (NER) can lead to error-prone repair. The ability of TFO to induce site specific mutations after systemic administration *in vivo* was recently demonstrated in a mouse model.⁹²

TFOs can also increase the level of recombination events in chromosomal and extra-chromosomal genes. The coupling of DNA cleaving agents to TFO leads to double-strand breaks which have been shown to induce recombination events.⁹³ The cellular DNA repair pathways appear to be involved in the process of triplex induced recombination through NER. The combination of a targeting TFO and recombination donor fragment has been used to demonstrate gene correction.

While the genome modification strategies have been shown to work in principle it has also become apparent that issues of delivery, in particular on the cellular level need to be resolved to achieve levels which are of potential therapeutic importance.⁸⁹

18.8 RNA INTERFERENCE (RNAi)

RNA interference (RNAi), the inhibition of gene expression by homologous double-stranded RNA (dsRNA), has become an important tool for the elucidation of gene function. Originally the phenomenon had been discovered as post-translational gene silencing (PTGS) in plants⁹⁴ and "quelling" in fungi⁹⁵ but the realization that this was a novel mechanism for gene silencing came later from experiments in *C. elegans.*⁹⁶ The effect is sequence specific and around ten times more potent than antisense as only a few molecules per cell of dsRNA are sufficient in silencing gene expression from the target RNA. The dsRNA can be administered by soaking the worms in it or by feeding of *E. coli* which express it. The effect can spread throughout the whole body and can also affect the germ line for several generations.⁹⁶

The similarities between sequence identity specific gene silencing found in plants (co-suppression, PTGS) and fungi (quelling) suggest that these mechanisms, including RNAi, probably form part of an ancient posttranscriptional gene regulation pathway: the introduction of homologous double-stranded DNA causes a knock-down of gene expression in insect cell lines but not mammalian cells. The mechanism of RNAi is evolutionarily conserved among eukaryotes and in addition to the response to exogenous NA it is also involved with the stabilization of the genome through the sequestration of repetitive sequences.⁹⁷

The proposed biological roles of RNAi include resistance to viruses, transposon silencing and regulation of endogenous gene expression, particularly during development. The introduction of the large (\sim 500 nt) dsRNA into the cytoplasm after transformation into smaller interfering RNA effector molecules (siRNA)⁹⁸ leads to a "sequence identity dependent" degradation of target mRNA which subsequently leads to gene silencing.

RNAi has become a valuable experimental tool for investigating gene function in *C. elegans*, *D. melanogaster*, and plants⁹⁹ but proved unsuitable for silencing in mammals. In this group of organisms the built-in defence mechanisms triggered by dsRNA of more than 30 nt (e.g., RNA-dependent protein kinase pathway which leads to nonspecific translation arrest by phosphorylation of EIF-2a).

The discovery that chemically synthesized small (21 to 23 nt) fragments of dsRNA which do not easily trigger this defence mechanism feed into the same mechanism as RNAi affords the opportunity to use these small interfering RNA (siRNA) to regulate gene expression in mammalian cells.¹⁰⁰

18.8.1 RNAI MECHANISM

RNAi degradation of mRNA starts with the processing of long dsRNA precursors by the Dicer type of nucleases to produce small 21 to 23 nt RNA fragments which are subsequently incorporated into the RNA-induced silencing complex (RISC) (Figure 18.11).



FIGURE 18.11 (See color insert) Model of the RNAi. Doubles stranded RNA is recognized and cleaved by the Dicer complex into small fragments which are then loaded onto the RISC complex where after unwinding a single RNA strand act as guide to direct cleavage of the target mRNA.

The Dicer enzyme is a member of the RNase III family which contains three classes of enzymes based on their domain structure: bacterial RNase III with a single catalytic domain and a dsRNA binding domain, the Drosha nucleases with dual catalytic domains, and finally the Dicer class which contains a dual catalytic domain in addition to helicase and PAZ motifs. The Dicer enzymes are evolutionary well conserved and recognize and process dsRNA to siRNA in, e.g., *Drosophila*, *C. elegans*, and mammals.⁹⁷ After processing by the Dicer enzymes the long double stranded RNA now consists of smaller fragments with 3' hydroxyl groups and symmetric 2 to3 nt overhang and 5' phosphate which interact with the RNA-induced silencing complex (RISC) (Figure 18.11).

The RISC complex represents a large protein-siRNA complex which targets RNA transcripts for degradation.¹⁰¹ In mammals a defence mechanism prevents use of dsRNA (RNAi) but siRNA can be loaded directly onto RISC.
The core components of the RISC complex are the siRNA and a number of proteins. The exact composition is still very much evolving but seems to involve AGO2, a member of the Argonaute gene family (co-purifies in Drosophila S2 cells), as well as RDE-1, RDE-4, and DRH-1.97 While binding of the siRNA is in the double-stranded form unwinding of the strands seems to be required¹⁰² vielding a single stranded antisense template to guide recognition of the target mRNA. The "ease" of unwinding determines where the unwinding through the helicase starts and also determines which of the double strands is actually loaded into the RISC to act as guide for the mRNA selection.¹⁰³ The RISC precursor complex (~250 kD) is transformed in the presence of ATP into the active 100 kD complex that can endonucleolytically cleave the substrate mRNA in regions homologous to the siRNA.¹⁰² The substrate is probably being identified through Watson-Crick base pairing with the siRNA. The endonuclease cleavage of the mRNA substrate only occurs in the homologous regions at a single side in the center of the mRNA siRNA duplex 10nt from the 5' end of the guide siRNA.¹⁰⁰ The cleavage of the target mRNA reduces the number of translation templates and thus expression of the corresponding gene. Non-perfect base pairing of the guide siRNA strand in the RISC complex and the target sequence does not induce mRNA degradation but translational repression, a mechanism of action commonly used by endogenous miRNA.

The persistence of RNAi mediated effects in *C. elegans* over several generations suggests that RNAi may also be involved in gene regulation at the transcriptional level most likely by epigenetic mechanisms¹⁰⁴ (also reviewed in Ref. 101).

18.8.2 SI RNA CHARACTERISTICS

RNAi based on long dsRNA is efficient as a number of siRNA which can effectively bind to the target mRNA emerge after "dicing." In the case of siRNA directed gene silencing the siRNA needs to be carefully chosen as different siRNA targeting the same mRNA will vary significantly in their ability to down regulate expression. Characteristic properties of siRNA include 5' phosphorylated ends, a 19-nucleotide (nt) double-stranded region and the 3' end with an un-phosphorylated 2-nt overhang. The process of sequence selection is largely empirical as no clear rules have been established so far. A number of guidelines have been proposed which were recently summarized in a review.¹⁰⁵ Not only is the activity of siRNA in mammalian cells related to the specific structure but also to mRNA secondary structure and protein binding. The requirements for siRNA are also distinct from those required for antisense oligonucleotides.¹⁰⁶ Favorable sites were correlated to those mRNA sequences which could be efficiently accessed and cleaved by RNase H.¹⁰⁷ Another important factor in the design of siRNA is the asymmetric loading of both strands into RISC; siRNA designed with these rules in mind have been shown to suppress their respective target at concentrations ~ 100 fold lower than those previously used.¹⁰³

In general siRNA have shown excellent sequence specificity differentiating even single nt differences,¹⁰⁸ but it has also been shown that the effects of siRNA on gene expression may not be sequence specific but actually siRNA specific as knock-down of nontarget genes with limited sequence overlap (> = 11 nt) has been observed.¹⁰⁹

The sequence specificity also appears to be important in determining the mode of action: while siRNA requires a perfect match to the target sequence and leads to target RNA degradation miRNA with several mismatches will inhibit specifically at the translation step.

18.8.3 MICRORNAS

MicroRNAs (miRNA) are small regulatory RNA first discovered in *C. elegans* where *lin-4* and *let-7* code for small RNAs which control development.^{110,111} A large number of other miRNA have since been identified in animals and plants underlining their potential importance for wider gene regulation.^{112,113} MicroRNAs are derived from large precursors (pre-miRNA) transcribed from noncoding genes. The precursors have self-complementary regions which allow the formation of fold back structures and are processed by the Dicer Type III RNase Drosha into smaller stem-loop precursors (pre-miRNA).¹¹⁴ These structures are then thought to be processed through Dicer into mature miRNAs. In animals the miRNA inhibit translation of similar (nonperfect base pairing) target sequences rather than guiding cleavage of perfectly matched RNA sequences (siRNA), in plants the miRNA mechanism of action is more similar to siRNA. Thus functionally the differentiation of miRNA and siRNA is largely dependent on the precision of annealing with the target sequence.¹¹²

18.8.4 RNAI PLASMID VECTORS

As mammals lack the RNAi amplification mechanisms found in plants and worms¹¹⁵ effects of synthetic siRNA tend to be short lived. RNAi expression from plasmid vectors potentially offers a strategy to allow longer-term modulation through RNAi.

The plasmid vectors produce transcripts modulating genetic regulation using either siRNA or modified miRNA to reduce expression of the target sequence, i.e., by transcript degradation or inhibition of translation, respectively. Both strategies have demonstrated the capability to inhibit expression of a broad variety of genes in many different cell types, suggesting the phenomenon to be universally applicable. The suppression of expression can reach levels of 80% to more than 90% but a basal expression tends to remain, making RNAi an important tool for gene "knock-down" (in contrast to "knock-out").¹¹⁶

Plasmid vectors expressing long hairpin under control of RNA polymerase II promoters have been shown to be effective in systems which lack the interferon response induced by long dsRNAs but were thought to be of limited utility in mammalian cells.¹⁰⁵ More recently expression for a long doublestranded RNA from a pol II promoter was used for gene knock-down in a murine model *in vivo*.¹¹⁷

Systems which express shorter hairpin RNA which subsequently can be processed by Dicer into a siRNA have been developed by several groups (e.g., Ref. 118). In contrast to conventional plasmid constructs the transcription is initiated using RNA polymerase III promoters, namely U6 and H1.

RNA polymerase III normally transcribes small non-coding transcripts and has a number of features that make it particularly suited as a promoter for the transcription of small siRNA constructs: transcription start and end are well defined by defined nucleotides, and the transcripts lack capping and polyadenylation at the 5' and 3' ends. These features allow the design of constructs that result in small RNAs with structural properties that resemble Dicer products and have been identified as being important for siRNA function, such as the 3' overhang of 2 to 3 nt, preferably two uridines.¹¹⁶ Properly designed shRNA can also take advantage of asymmetric loading of both strands into RISC.¹⁰³ Recently a system that allows tetracycline inducible expression from a modified U6 promoter has been reported.¹¹⁹

An alternative strategy to the use of inverted repeats and loops sequences to form short hairpin RNA (shRNA) is the co-expression of duplex forming sense and anti-sense strand from tandem reporters.¹²⁰ Transcription of the sense and antisense sequence is from two promoters placed in sequence or on two separate constructs to combine into a duplex RNA *in vivo*. This strategy does not rely on dice for the processing but it has been suggested that Dicer processing may make the RISC dependent elements more efficient.¹²¹

The use of miRNA expressed from a miR-30 pre-miRNA backbone was successfully used to induce post-transcriptional inhibition of HIV-1 replication.¹²² In contrast to the RNA degradation of siRNA the inhibition of gene expression by miRNA appears to occur at the level of translation.¹¹³ The difference in mechanism has been linked to the extent of complementarity with the target sequence; whereas siRNA show a perfect match with the target the miRNA sequences contain multiple mismatches.¹¹²

The potentially excellent sequence specificity combined with sustained expression from plasmids vectors makes RNAi expression vectors potentially suited for the therapy of genetic disorders which require the elimination or "knock-down" of expression from dominant genes (e.g., amyotrophic lateral sclerosis (ALS), spinobulbar muscular atrophy (SBMA)), oncogenes in cancer, or expression of viral genes.¹¹⁶

18.9 CONCLUSIONS

Clearly therapeutic ONs have demonstrated great therapeutic potential in the laboratory and in some cases in the clinic.⁶⁷ Nevertheless there remain a number of issues that need to be addressed in order to facilitate the broader

clinical application of some of these technologies. One of those is efficient and specific delivery of the ON to the target cells, and to the appropriate cellular compartment.³⁴ In general free ONs are being taken by a combination of adsorptive and fluid phase endocytosis which subsequently requires release from the endosomal-lysosomal compartment and delivery strategies may be required to increase the efficiency of these steps. A further problem is the limited ability to accurately predict accessibility of target sequences in mRNA for hybridization caused by the formation of secondary structures or the binding to proteins. The binding of ONs to cellular proteins has also been linked to some of the non-specific or off site ON effects which sometimes have blurred the specific effects (e.g., Ref. 21). The efficacy of strategies which aim to knock down gene expression through mRNA degradation steps will to some extend depend on the mRNA metabolism, i.e., mRNA expression levels and half-life, which can range from a few minutes to several hours. Many of the questions relate to the fundamental mechanism of gene regulation. The NA discussed in this chapter also have an important role to play in linking genetic information from the human genome project to gene function and the identification of potential new disease-related NA targets. The challenges encountered in developing such insights into genetic medicines are complex and need to be addressed in a multidisciplinary framework which brings together chemistry, molecular biology, delivery, and pre-clinical and clinical development expertise.

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19 Antisense and Antigene Oligonucleotides: Structure, Stability and Delivery

Ram I. Mahato, Zhaoyang Ye, and Ramareddy V. Guntaka

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Acknowledgment

References

19.1 INTRODUCTION

Diseases are being understood and treated at an increasingly higher level of genetic order and function. Recent advances in nucleic acid-based therapeutics offer great hope in controlling sequence-specific aberrant gene expression at the level of transcription or translation. Oligonucleotides (ODNs) which control gene expression at translation levels are known as antisense ODNs, whereas those act at transcriptional levels are triplex-forming oligonucleotides (TFOs), called anti-gene strategy (Figure 19.1). There has been tremendous progress in the understanding and application of antisense ODNs since first proposed in 1978 by Zamecnik and Stephenson.¹ A number of different ODNs have been in clinical trials against many diseases, such as human immunodeficiency virus HIV infections and cytomegalovirus (CMV) ocular infections as well as in the control of hematological disorders including Crohn's disease.² Several antisense ODN-based formulations are in clinical trials and there is already one FDA approved product for the treatment of human CMV-induced retinitis.³ However, triplex forming oligonucleotides (TFOs), which can inhibit gene transcription are quite promising antigene therapy for modulation of gene expression.^{4–6}

The length of ODNs is determined based on antisense mechanism and other practical considerations. Antisense ODNs are typically synthesized in lengths of 13 to 35 nucleotides. The minimum ODN size needed to recognize a specific gene is between 12 and 15 bases in length. It is estimated that at least 12 bp need to form a stable hybrid with a phosphodiester backbone at physiologic conditions.

In addition to antisense and antigene ODNs, antisense RNA, ribozymes, and small interfering RNA (siRNA) can also be used for inhibition of gene



FIGURE 19.1 Strategies for modulating gene expression.

expression. Antisense RNA strategy relies on the transfection and subsequent expression of a plasmid carrying the cDNA of the gene of interest subcloned into the vector in an antisense orientation.⁷ The expressed antisense RNA which is capable of hybridizing exclusively with the mRNA of the gene of interest will thus block protein synthesis. Ribozymes, also known as catalytic RNA, is an RNA molecule having catalytic enzyme activity to cleave single-stranded RNA in a sequence-dependent manner.⁸ RNA interference (RNAi) is the sequence-specific gene-silencing induced by double-stranded 21 to 23 nucleotide long small interfering RNA (siRNA).⁹ Antisense RNA, ribozymes and siRNA are also quite promising and these strategies are discussed in a separate chapter by Andreas Schatzlein.

It is a challenging task to specifically deliver oligonucleotides to target cells in various organs and tissues. The following problems associated with antisense ODNs and TFOs should be addressed before they can be used as pharmaceuticals: (i) in vitro and in vivo stability of ODNs. Native phosphodiester ODNs are subject to rapid degradation by serum and cellular nucleases from the 5' and/or 3' terminus.^{10,11} To overcome this, many structural modifications have been made on their backbones to improve their stability so that they can reach their targets in intact forms.^{12,13} (ii) Due to their polyanionic properties, the nucleic acids are taken up by the cells by a combination of fluid-phase (pinocytosis), adsorptiove and receptor mediated endocytosis.¹⁴ However, the uptake of free ODNs into various organs in the body is still not efficient to provide therapeutic benefits. Many strategies have been proposed to improve their cellular uptake including the use of liposomes,¹⁵ polymeric carriers,¹⁶ or by direct conjugation with carrier molecules, such as lipids,¹⁷ hydrophilic molecules,¹⁸ and fusogenic peptides.^{19,20} These delivery strategies can improve their ability to interact with cellular membranes. In this chapter, we will discuss in depth the opportunities and challenges of antisense ODNs and TFOs, with main focus on their delivery issues.

19.2 STRUCTURAL MODIFICATION OF OLIGONUCLEOTIDES

Both antisense ODNs and TFOs must reach their targets intact to modulate gene expression by binding to mRNA to form DNA–RNA hybrid duplex or to genomic DNA to form triplex inside nucleus in the cells. Enormous amount of literature exist on improving the stability of these ODNs. Modifications have been in all the relevant subunits, such as sugar, base and phosphate backbone (Figure 19.2). Some of these strategies are illustrated in Figure 19.3 and will be discussed below.

19.2.1 FIRST-GENERATION OLIGONUCLEOTIDES

Endonucleases and exonucleases attack DNA molecules at the phosphodiester bridges and break them down to mononucleotides. Therefore, the *first-generation ODNs* were designed to make the internucleotide linkages more



FIGURE 19.2 Different possibilities for modification of oligonucleotides. Modifications are undertaken in all the relevant subunits, i.e., sugar, base and phosphate backbone.

resistant to this attack. This is accomplished primarily by replacing one of the nonbridging oxygen atoms in the phosphate group with either a sulfur or a methyl group. The former modified forms are called phosphorothioate oligonucleotides (PS ODNs) and the latter as methylphosphonate ODNs (MP ODNs), respectively.² MP ODNs are neutral in charge and lipophilic in nature. In addition to being nuclease resistant, MP ODNs are usually taken up by cells in a more efficient manner. However, these modified ODNs have not been widely used, probably because (i) they are poorly soluble in water and (ii) they do not activate RNase H-mediated cleavage of the mRNA to which the molecule may be hybridized, resulting in loss of significant antisense effect.

PS ODNs are by far the most extensively studied and are in human clinical trials for treatment of various diseases.²¹ PS ODNs have a half-life of approximately 9–10 h in human serum compared to \sim 1 h for unmodified ODNs.^{22,23} In addition to nuclease resistance, PS ODNs form regular Watson–Crick base pairs, activate RNase H in the DNA–RNA hybrid duplex, and display attractive pharmacokinetic profiles.²⁴ However, the substitution of nonbridging oxygen by sulfur brings several limitations. PS ODNs have chiral centers at the internucleoside phosphorothioates and thus have



FIGURE 19.3 Chemical modification of oligonucleotides for their improved stability and activity. (A) Backbone modification; (B) sugar modification.

slightly reduced affinity towards complementary RNA molecules in comparison to the corresponding PO ODNs. They bind to key structural proteins, enzymes, receptors, growth factors, and transcription factors via ionic or hydrophobic interactions with their modified backbones.^{25,26} High-doses of PS ODNs into rodents and non-human primates have shown to be associated with acute hemostatic changes, complement activation and/or coagulation activities, acute renal failure and/or thrombocytopenia.^{27–29} The lower doses of PS ODNs used for clinical trials in humans, however, were generally well tolerated.

19.2.2 SECOND- AND THIRD-GENERATION OLIGONUCLEOTIDES

A number of strategies have been introduced to overcome the limitations of PS ODNs, while preserving their useful properties. Using end-capped PS, where both 3' and 5' ends are phosphorothioated, appears to be a reasonable compromise in that exonuclease stability is conferred on the molecules and side effects associated with fully phosphorothioated molecules are significantly reduced.³⁰ Phosphorothioates are often combined with other modifications, especially phosphorothioate 2'-O-methyl-ODNs and MP ODNs to develop mixed-backbone oligonucleotides (MBOs).³¹ These MBOs have all the required properties for antisense activity with minimal polyanion-related

effects and are now commonly known as *second-generation antisense* ODNs.³² End-modified MBOs, in which other modifications are incorporated at the 3'-end or at both the 3'- and 5'-ends of PS ODNs, have shown improved specificity, biological activity, *in vivo* stability, pharmacokinetic and safety profiles, lower polyanion-related effects, and reduce protein binding. Centrally-modified MBOs, in which other modifications are in the center of the PS ODNs, show increased binding affinity, increased RNase H activation and consequently rapid degradation of RNA compared with end-modified MBOs. These MBOs showed improved pharmacokinetic and safety profiles.³²

DNA and RNA analogues with modified phosphate linkages or riboses as well as nucleotides with a completely chemical moiety substituting the furanose ring have been developed. These molecules are known as third generation antisense ODNs.¹³ N3' \rightarrow P5' phosphoramidates are third generation antisense ODNs, in which the 3'-oxygen-bridging has been replaced with amino (-NH) group). $N3' \rightarrow P5'$ phosphoramidate ODNs form stable duplexes with both complementary DNA and RNA. However, unlike PS ODNs, $N3' \rightarrow P5'$ modified ODNs are unable to activate RNase H when bound to complementary RNA.³³ Despite inability to activate RNase H, $N3' \rightarrow P5'$ phosphoramidate ODNs have shown potent antisense activity in both cell culture³³ and in vivo.³⁴ There is a sequence-specific inhibitory effect on expression of the corresponding proteins. The survival rate of the mice treated with $N3' \rightarrow P5'$ modified ODNs was significantly higher than PS ODNs or control mismatch ODNs.³³ Furthermore, $N3' \rightarrow P5'$ phosphoramidate homopyrimidine strands form more stable triplexes with both dsRNA than corresponding native DNA 12,35

However, phosphoramidates do not efficiently transport across cell membranes and do not activate RNase H. This creates a highly nucleaseresistant molecule with an ability to form very stable duplexes with singlestranded DNA, and RNA, by Watson–Crick base paring. They can also form stable triplexes with double-stranded DNA under near physiological conditions. Although the ability of the phosphoramidates to activate RNase H is weak, they effectively block translation because of the stability of the DNA/ RNA hybrids formed.

In addition to modifications to the internucleoside bridge, the 2'-deoxy- β -D-ribofuranose unit of the DNA backbone is another site for ODN modification (Figure 19.3). Various sugar modifications have been introduced into antisense ODNs to enhance binding affinity and nuclease resistance. Changing the sugar's glycosidic linkage from the naturally occurring β form to the α anomeric form increases the nuclease stability and compromises hybridization stability and ability to activate RNase H. Sugars are also typically modified at the 2' position with O-methyl, fluoro, O-propyl, O-allyl, or other groups.^{36,37} These modifications have been shown to increase affinity for RNA and impart some nuclease stability. Nevertheless, these molecules do not support RNase H activity and, for this reason, do not appear to have significant antisense activity in some assays.

19.2.3 MORPHOLINO OLIGOMERS

ODNs with morpholino nucleoside linked together by phosphorodiamidate groups are called phosphorodiamidate morpholino oligomers (PMOs) (Figure 19.4A). PMOs are cheaper and more efficiently assembled than many of any other modified ODNs.³⁸ The chemistry of PMOs is different from DNA in that the deoxyribose sugars are replaced with a six-membered morpholine ring and the phosphodiester linkages are replaced with nonionic phosphorodiamidate linkages. PMOs have good aqueous solubility, are extremely stable to degradation in biological fluid³⁹, but are sensitive to degradation after prolonged exposure to low pH. Despite possessing a chiral phosphorodiamidate linker, PMOs form heteroduplexes with complementary RNA which are more stable than the corresponding native DNA : RNA duplexes.

PMOs have been shown to be more effective antisnese agents than PS ODNs in cell-free systems and in various cultured cells using the scrape-loading method to allow for efficient cellular uptake.⁴⁰ Summerton et al. (1997)⁴⁰



FIGURE 19.4 Morpholino oligomers. A) Conversion of ribonucleoside to phosphoramidated-linked morpholino oligomers (PMOs); (B) Sequence specificity of PMOs and PS ODNs. Reproduced from Summerton et al. (1997) *Antisense Nucleic Acids Drug Dev.*, **7:** 63–70, with permission.

demonstrated that PMOs are highly effective and have specific antisense activity over a concentration range more than two orders of magnitude greater than the concentration range wherein the corresponding PS ODNs achieved reasonable efficacy and specificity (Figure 19.4B). This is attributed to the fact that PMOs do not bind to nonspecific proteins as PS ODNs, and also are much more sequence specific, because they depend on a steric blocking mechanism rather than activating RNase H, which can result in cleavage of many partially complementary sequences other than the target sequence. Even in the case of their poor cellular uptake, PMOs, specific for the tumor necrosis factor- α (TNF- α) in mouse macrophages, were shown to be more effective than PS ODNs.⁴¹ PMOs could induce missplicing, in addition to interfering with pre-mRNA splicing. More recently, a 28-mer PMOs targeted to c-mvc pre-mRNA, overlapping the initiation codon, was shown to completely inhibit c-MYC protein expression in several human leukaemia cell lines, following treatment with streptolysin O. In addition to inhibiting splicing, mis-splicing of the c-myc pre-mRNA gave rise to a translatable mRNA which resulted in a shorter, N-terminal deleted, MYC protein. In contrast, a fully modified 2'-methoxyethoxy ODN, which has high affinity for complemetary RNA and is also incapable of activating RNase H, did not result in inhibition of translation, splicing or induce mis-splicing of c-mvc pre-mRNA in the same cell lines. Caliciviruses infect and cause disease in animals and humans. PMO can disrupt caliciviral gene function in a nucleic acid sequencespecific manner and are potentially effective antiviral agents⁴².

The cytochrome P450 (CYP) family is the most catalytically versatile component of the phase I oxidation metabolic pathway. Cytochrome P450 participates in the metabolism of a large majority of drugs used in clinical practice. The inhibition of specific enzymes of this family can significantly alter the disposition and toxicity of substrate drugs by reducing and/or redirecting their metabolism. There is one review discussing the use of PMOs in CYP inhibition.⁴³ The use of PMO strategies to target CYP enzymes may result in safer and more uniform therapeutic applications. Inhibition of cytochrome P450 (P450) 3A4 expression using AVI-4557, a 20-mer PMO, was examined in primary human hepatocytes and in Caco-2 cells.⁴⁴ AVI-4557 inhibited expression of CYP3A4 in Caco-2/h3A4 cells by 64% at 24h following administration of 2.8 µM by an assisted delivery protocol. Inhibition of CYP3A activity was observed in primary human hepatocytes after 24h exposure to AVI-4557 by an average of $32 \pm 11\%$. Furthermore, AVI-4557 exposure resulted in a sequence-dependent inhibition of cyclophosphamiderelated cytocidal activity and a sequence-dependent induction of paclitaxolrelated cytocidal activity in both cell types.

The PMOs have significant advantages over other ODN anologs from the perspective of oral drug delivery. In one study, the oral fractional bioavailability of two distinct PMO sequences (AVI 4126 and AVI 4472) targeted to c-myc and cytochrome P-450 (CYP) 3A2, repectively, were investigated. After oral administration, 78.8% of the dose (3 mg/kg) of AVI 4126 was detected in plasma over 10 min through 24 h. And a sequence specific reduction of the target protein in the liver, however, a classic dose-response inhibition was not observed. AVI 4472 caused a sequence specific reduction of approximately five-fold in the rat liver CYP3A2 protein levels after oral administration.⁴⁵

In a preclinical mouse model,⁴⁶ a 20-mer PMO complementary to the hepatitis C virus (HCV) internal ribosome entry site (IRES) was shown to inhibit the luciferase reporter mRNA expression by greater than 95% for at least 6 days and the antisense effect was specific and dose dependent. To increase the cellular uptake of PMOs, the HIV Tat peptide was attached to the PMOs.²⁰ Fluorescence was seen in 100% of HeLa cells treated with Tat-PMO-fluoresce in conjugate. Most Tat-PMO conjugate was associated with cell membranes, and internalized conjugate was localized in vesicles, cytosol, and nucleus. Tat-PMO conjugate targeted to c-*myc* mRNA downregulated c-*myc* reporter gene expression with an IC50 of 25 μ M and achieved nearly 100% inhibition. Tat-PMO conjugate targeted to a mutant splice site of beta-globin pre-mRNA dose-dependently corrected splicing and upregulated expression of the functional reporter gene. However, Tat-mediated PMO delivery required higher concentrations of PMO (>10 μ M) to cause antisense activity and caused some toxicity.

19.3 MECHANISMS OF ACTION OF ANTISENSE OLIGONUCLEOTIDES

Antisense ODNs mostly work at post-transcription levels. Normally, transcription of any given gene is carried out by the RNA polymerase II from a transcription start site to give rise to heteronuclear RNA, which is subsequently processed by splicing and polyadenylates to mature mRNA that is trafficked to the cytoplasm where they get translated into proteins. Therefore, antisense ODNs are designed to bind to their target sense RNA sequence through the formation of reverse complementary (antisense) strands with the mRNA. The two most likely mechanisms of inhibition of the gene expression by antisense oligonucleotides appear to be (i) direct blocking in pre-mRNA and/or mRNA of sequences important for processing or translation, and (ii) degradation of the RNA transcript by RNase H at the site of oligonucleotide binding (Figure 19.5).^{47,48} RNase H cleaves the RNA component of RNA : DNA hybrids and is abundant in the cytoplasm and nucleus of a large number of organisms.

The mechanism of action of antisense ODNs appears to be more complex than originally thought. Events that are triggered as a result of heteroduplex formation are dependent on the nature of the antisense molecules used for mRNA targeting. ODNs of many, but not all, types support the binding of endogenous RNase H at sites of RNA–DNA duplex formation.^{49,50} Such binding is thought to be an important effector of antisense actions because once bound, RNase H functions as an endonuclease that recognizes and cleaves the RNA moiety in the hybrid (Figure 19.5). Of significant interest also is the fact that the DNA comprising the duplex is undamaged by the



FIGURE 19.5 Mechanisms of action of antisense oligonucleotides. (A) Translational arrest by blocking the ribosome; (B) Degradation of mRNA by RNase H cleavage.

enzymatic attack. Therefore, it is free to hybridize with multiple RNA molecules, leading to their destruction in a catalytic manner.

PS ODNs are thought to activate RNase H efficiently, while MP ODNs do not support the activity of this enzyme at all. PO, PS and MP ODNs hybridize to pre-mRNA in a sequence specific manner.⁴⁸ Duplexes formed with PO and PS ODNs are susceptible to cleavage by RNase H, while RNA in the duplexes formed with an MP ODNs is resistant to cleavage by RNase H. The resistance of MP ODNs to RNase H decreases with the decrease in the number of methylphosphonate deoxynucleotides in the MP ODNs.⁴⁸ Eukaryotic RNase H generally requires the DNA portion of the duplex to have five or six consecutive internucleotide linkages that can be recognized by RNase H.^{47,51} Keeping this in mind, ODNs are often synthesized to contain with nuclease resistant modifications at the 3' and 5' ends of the oligo, and six to eight unmodified or phorothioate modified linkages in the central portion. These chimeric ODNs inhibit 3' and 5' exonuclease degradation, while still serving as a substrate for RNase H.^{47,51} RNase H may produce unanticipated, nonsequence-dependent effects by cleaving transiently formed duplexes, or with sites of partial complementarity.

Antisense ODNs containing four contiguous guanosine residues should not be employed as they may lead to G-quartet formation via Hoogsteen basepairing that can decrease the available ODN concentration and might result in undesired side-effects. Modified guanosines (e.g. 7-deazaguanosine, which cannot form Hoogsteen base pairs) may be used to overcome this problem.¹³

19.4 CpG MOTIFS AND IMMUNOSTIMULATION

Most antisense ODNs contain dinucleotide sequence 5'-cytosine guanosine-3', commonly known as CpG motif. These CpG motifs can illicit an immune reaction.^{52,53} However, methylation of the cytosine in CpG dinucleotide completely abrogated any immunostimulatory properties, confirming that the immunostimulatory properties of these ODNs were dependent on the presence of the unmethylated CpG dinucleotides.⁵⁴ As discussed in detail by Krieg and associates,⁵⁵ CpG motif containing ODNs have been shown to induce (Figure 19.6):

- Toll-like receptor 9 (TLR9) to initiate signaling pathways that activate several transcription factors, including NF-κB and AP-1;⁵⁶
- the direct activation of murine and human B cells, resulting in IL-6 and IL-10 secretion, major histocompatibility complex (MHC) class II and B-7 upregulation and resistance to apotosis;
- the direct activation of macrophages, dendritic and antigen presenting cells, resulting in the release of IFN-γ and IL-12;
- the activation of NK cells, resulting in the rapid induction of IFN- γ ; and
- the indirect influence on the activation of CD4 and CD8 T cells.



FIGURE 19.6 Structure and functions of CpG motifs. (A) Structure; (B) role of CpG motifs in immunostimulation.

Therefore, CpG motif containing ODNs are being explored as immunomodulators in antiviral, antibacterial, anticancer, and anti-inflammatory therapies.⁵⁷ Following intratumoral injection, CpG containing ODNs abolish the immune privilege of tumors by recruiting and activating local dendritic cells and inducing IL-12 production. Therefore, CpG containing ODNs can be used for cancer treatment.⁵⁸ Several studies have demonstrated the potential effectiveness of CpG containing ODNs as therapies in animal models, and a number of CpG containing ODNs are currently being tested in clinical trials.^{59,60} It is clear that if a selected antisense ODN sequence has a CpG motif, extreme care must be taken in establishing its specificity of antisense activity.

19.5 TRIPLEX FORMING OLIGONUCLEOTIDES (TFOs)

In contrast to antisense ODNs, TFOs inhibit gene transcription by forming DNA triple helices in a sequence-specific manner on polypurine : polypyrimidine tracts.⁵ Targeting ODNs to the gene itself presents several advantages as compared to *antisense ODNs* which are directed to mRNAs or inhibiting the binding and catalytic activity of even more protein molecules using *conventional drugs* are less efficient ways of suppressing protein activity.^{61,62} There are only two copies (two alleles) of the targeted gene whereas there are thousands of copies of an mRNA. Blocking mRNA translation even by inducing sequence-targeted cleavage of the RNA does not prevent the corresponding gene from being transcribed, thereby repopulating the RNA pool. In contrast, prevention of gene transcription is expected to bring down the mRNA concentration in a more efficient and long-lasting way.

Inhibition of gene transcription depends on the residence time of the TFO on its target sequence as well as its nuclease sensitivity. One of the difficulties in designing a TFO resides in the accessibility of the target sequence in the chromatin structure of the cell nuclei. DNA normally exists in a duplex form, but under some circumstances, DNA can assume triple helical (triplex) structures, which are either intramolecular or intermolecular. Intermolecular triplexes are formed by the addition of a sequence-specific third strand to the major groove of the duplex DNA.⁶³ Triplex formation may then prevent the interaction of various protein factors required for transcription, or it may physically block the initiation or elongation of the transcription complex.

TFOs are of 10–30 nucleotides in length and require runs of purines on one strand and pyrimidines on the other for stable hybridization. TFOs may consist of either pyrimidine bases to form the $Py \cdot Pu^*Py$ triplex or predominantly purine bases to form $Py \cdot Pu^*Pu$ triplexes, depending on the nature of the target sequences (Figure 19.7). TFO bases form reverse Hoogsteen bonds only with adenines and guanines, thus forming base triads. An A or a T in the TFO can bond with the A of an A:T pair in the DNA duplex, while G can bond with the G of a G:C pair. C can also bond with the G of a G:C pair after protonation at the N3 position in slightly



FIGURE 19.7 Pyrimidine and purine motifs for triple helix formation.

acidic media (pH < 6). Depending on the relative orientation of the third strand (R), two types of triplex structures can be formed. In the pyrimidine (or Y : R : Y) motif, a homopyrimidine oligonucleotides binds in a direction *parallel* to the pruine strand by Hoogsteen hydrogen bond, with canonical base triplets of T : A : T and C : G : C. In the alternate purine motif (R : R : Y), a homopurine strand binds *antiparallel* to the purine strand by reverse Hoogsteen hydrogen bonds, with base triplets of A : A : T and G : G : C).^{5,64} In addition, a (G, T)-motif TFO is also permitted, whose orientation depends on both the number of GpT or TpG steps and the length of G and T tracts. Some TFOs have been designed to "switch" strands when blocks of homopurine sequences alternate between strands at the duplex DNA target sites. Based on the studies of TFOs performed to date, it appears that in the absence of chemical modification, a TFO sequence should contain at least 20 bases in order to bind its target site with sufficient affinity so as to achieve biochemical effects.

Guanine-rich TFOs can form intra- or inter-molecular four-stranded structures involving G-quartets; these structures are favored when the sequences contain repeats of consecutive guanines. (G, A)-TFO can form other intermolecular structures, such as parallel homoduplexes involving A·A, G·G, and G·A base pairs. Monovalent cations, such as K⁺ present under physiological conditions, enhance the formation of quadruplexes, but decrease the formation of a parallel homoduplex in the presence of divalent cations.⁶⁵ To overcome this K⁺ effect, several strategies have been employed. In one, some of the G residues within a G-rich TFO were replaced with 6-thioguanine.

Although this modification reduces G-quartet formation, it also lessens the overall binding affinity of the third strand. Similarly, the replacement of the N-7 of guanine with carbon, creating 7-deazaguanine, eliminates the ability of the TFO to form G-quartets but also decreases the capacity of the oligonucleotide to form triple helices.⁶⁶ Cytosine-rich TFO can form, under acidic pH conditions, four-stranded structures called i-DNA involving hydrogen-bonded pairs between cytosine and protonated cytosine (C·C⁺).

19.6 STABILITY OF TRIPLEX DNA

19.6.1 BARRIERS TO FORMATION AND STABILITY OF TRIPLEX DNA

Triplex formation involves the binding of a negatively charged third strand to a double negatively-charged duplex. Neutralization of charge repulsion is typically provided experimentally by levels of Mg^{2+} ions (5–10 mM) that are much higher than what is thought to be available in cells.⁵ In contrast, monovalent cations (Na⁺, K⁺) at physiological concentrations (140 mM) inhibit triplex formation and favor G-quartet formation. Furthermore, triplex formation involves conformational changes on the part of the third strand, and some distortion of the underlying duplex. Pyrimidine motif triplexes are unstable at physiological pH because of the requirement for cytosine protonation that occurs at relatively acidic pH (pK_a=4.5). Pyrimidine motif triplexes containing adjacent cytosines are often less stable than those with isolated cytosines. In addition, purine motif third strands (which are G rich) may form G tetrads in physiological levels of K⁺, which inhibit triplex formation. All these factors impose kinetic barriers on triplex formation and reduce the stability of triplexes once formed.⁵

19.6.2 STRATEGIES TO IMPROVE TRIPLEX DNA STABILITY

Binding constants on the order of 10-100 nM are likely required for efficient TFO activity in cultured cells and *in vivo*. Considerable research efforts are presently devoted to increasing the stability of triple helices under physiological conditions. These include overcoming the pH dependence in C containing TFO, substitution of thymines in TFOs to avoid the less stable T · A*T triplet, and minimization of self-associated-structures which compete with triplex formation, especially in the case of G-rich or C-rich TFOs.

Neutralization of electrostatic repulsion among DNA strands associated in triplex may increase triplex forming efficiency. In this regard, Ferdous et al. introduced comb-type copolymers of poly(L-lysine) with polysaccharide to stabilize the triplex,⁶⁷ which were shown to form reversible complex with TFO.⁶⁸ The T_m value of DNA triplex was increased up to 15°C in the presence of this comb-type copolymer depending on the grafting degree of dextran chains. The negative effect of K⁺ on triplex formation was almost completely abrogated in the presence of the copolymer.⁶⁹

Several intercalating agents have been developed, which can stabilize different triplets. Intercalating agents are often conjugated to the 5'- or 3'-end or to internal positions to stabilize triplexes, especially those containing one or two base interruptions in the purine rich TFOs. Some chemical agents are attached to TFOs to introduce irreversible modifications on the DNA targets. For example, psoralen and orthophenanthroline derivatives are remarkable by their in vitro efficacy at inducing triplex-mediated cross-linking and cleavage reactions, respectively, on a duplex target, such that a durable biological effect can be obtained.⁷⁰ Several modified bases have been substituted for cytosine in order to extend formation of homopyrimidine oligonucleotide-directed triplexes under the physiological pH ranges. Some modifications intended to increase ODN nuclease stability such as changing the anomeric configuration of glycosidic bond from β to α do not disturb the triplex stability. Substitution of deoxyribose for ribose in the TFO can stabilize triplexes for the (C, U) or (G, U)-motif in a parallel orientation with respect to the purine target sequence in duplex DNA. The stability of triplexes involving 2'-O-alkyl analogues is even higher than that of oligoribonucleotides. Backbone modifications, such as phosphoramidates and PNAs have also been exploited. Analogues containing $N3' \rightarrow P5'$ phosphoramidate linkages highly stabilize triplexes formed with (T, C)- and (T, G)motif TFOs.⁷¹ However, triplex stability is greatly reduced in the pyrimidine motif using phosphorothioates, whereas purine motif by this modification appears at most as stable as PO.

19.7 INHIBITION OF TRANSCRIPTION BY TRIPLEX FORMATION

TFOs have to reach the nucleus and compete with all proteins binding to DNA. Accessibility of target sites in the context of chromatin is crucial for the development of the anti-gene strategy. Transcriptionally active genes might be accessible to triplex-mediated regulation, especially when the target sequences for ODNs are located in the same DNA domain as transcription factor binding sites. TFOs bind efficiently to $Py \cdot Pu$ tract. If the $Py \cdot Pu$ tracts are localized in the coding regions of the gene, TFO binding presents a physical block to the progressing RNA polymerase that cannot unwind the triplex. This results in inhibition of mRNA synthesis and therefore synthesis of the functional protein. If a TFO binds in the promoter region, several events may occur that inhibit or activate gene expression. Other enzymes involved in transcription, replication, recombination, and repair processes, such as helicases and topoisomerases can also be disturbed.

Sequence-specific effects can be obtained by mechanisms other than the expected one. This is easily explained by the fact that some TFOs adopt highly structured conformations that can be recognized by specific proteins; G-rich TFOs are particularly prone to such effects. In most of the reports, control TFOs of different length or composition, or with disrupted tracts of

 $(G)_n$ are not satisfactory and definitive demonstration of a triplex-mediated mechanism is generally lacking. The best control experiment actually consists of using the same TFO but a mutated target sequence affecting triplex stability but not gene expression. However, this is easy to achieve when the target gene is carried by a plasmid but more difficult with endogenous genes. For example, the c-myc oncogene was first shown to be modulated by a TFO forming triplexes in the promoter region; however, a mechanism other than triplex formation was subsequently proposed: titration of one of the transcription activator by the purine-rich TFO could fully account for the observed decrease of c-myc RNA transcription. Recently, a covalent triplex was reported to enhance recombination processes between two tandem *supF* genes present on a plasmid construct, in mammalian cells.

19.8 PHARMACOKINETICS OF OLIGONUCLEOTIDES

The biodistribution and subcellular distribution of ODNs have been studied intensively in rodents and nonhuman primates. For their action at a target site within the body, there are many biological barriers to be overcome: moving from the bloodstream into tissues, from the extracellular space, across the plasma membrane and into the cytoplasm, and from the cytoplasm into the nucleus. To construct strategies of establishing efficient and safe delivery systems for ODNs, a thorough understanding of their *in vivo* disposition characteristics is necessary.

Unmodified phosphodiester (PO) ODNs were rapidly degraded by serum and cellular nucleases. The extent of radioactivity accumulated in the kidney for PS ODN samples was much lower than PO samples, possibly due to the extensive binding of PS ODNs to the plasma proteins, resulting in enhanced hepatic uptake but poor kidney uptake.¹⁰ Following intravenous administration, ODNs are rapidly eliminated from circulation and are widely distributed to most peripheral tissues, with liver and kidney accumulating the most.^{72–74} The pharmacokinetic properties of macromolecules after intravenous administration can be analyzed on the basis of a physiochemical model as described by Takakura and Hashida.⁷⁵

The disposition of ODNs at the whole body level can essentially be represented by hepatic uptake and urinary clearances, as illustrated in Figure 19.8. Urinary excretion is mainly dependent on the molecular weight of a compound, while its apparent charge affects both the hepatic uptake and renal excretion. Positively charged macromolecules have wide hepatic uptake and renal clearances. Larger hepatic uptake clearance is observed for strongly anionic compounds.^{75,76} Based on their studies, ODNs have been shown to rapidly eliminate from the circulation and are widely distributed into most of the tissues tested, with the liver, kidney, bone marrow and muscle accumulating the most. PS ODNs, possibly due to the extensive binding to the plasma proteins, accumulated much less in the kidney compared to PO ODNs. Takakura et al. (1996) examined the hepatic



FIGURE 19.8 Effect of backbone modification and carrier systems on the hepatic uptake and urinary clearances of oligonucleotides (ODNs) after systemic administration in mice. PO ODNs, 20 mer phosphodiester oligonucleotides; PS ODNs, 20-mer phosphorothioate oligonucleotides; Gal-PLL, galactosylated poly(L-lysine); BSA, bovine serum albumin; pCAT, plasmid DNA encoding chloramphenicol acetyltransferase; Dex (T-10), carboxymethyl dextran-thymidylic conjugate.

disposition characteristics of 20-mer PO ODNs and its partially (PS₃ ODNs, in which three internucleotide linkage at the 3'-end are phosphorothioated) and fully phosphorothioated (PS) derivatives in the isolated rat liver perfusion system after bolus injection into the portal vein. The magnitude of the hepatic interaction of ODNs increased as the extent of PS modification in the molecules increased: About 20, 36, and 52 of the injected dose was taken up by the liver during a single passage after bolus injection of PO, PS₃, and PS, respectively.⁷⁴

For further studies, we selected PS₃ which was taken up by both liver parenchymal and nonparenchymal cells. Coadministration of polyanions such as dextran sulfate, polyinosinic acid (poly[I]), polycytidic acid (poly[C]) and 4-acetamido-4'-isothiocynaostilbene-2,2'-disulfonic acid (SITS), caused substantial decrease in the amount of total recovery in the liver. It is well known that poly[I] binds to the scavenger receptors, whereas poly[C] does not.^{77,78} This suggests that PS₃ was nonspecifically taken up by the liver, as both small and large anionic molecules inhibited its hepatic binding. There are also some reports suggesting that PS are mainly taken up by the liver endothelial cells, and to some extent by hepatocyte and Kupffer cells.^{79,80} Sawai et al. (1996) clarified the renal disposition characteristics of³² P-labeled 20-mer ODNs using the isolated rat kidney perfusion experimental system.⁷² Binding of the ODNs to bovine serum albumin (BSA) in the perfusate prior to kidney perfusion was in the following order of magnitudes: $PS > PS_3 > PO$. ODNs showed a sulfur-atom dependent interaction with renal vasculature and hence the volume of distribution (V_d) increased as the extent of PS modification increased. A significant amount of the ODNs filtered through glomeruli underwent tubular reabsorption. Reabsorption might be mediated by the interactions with specific proteins in the brush border membrane. PS ODNs are accumulated in a non-filtering kidney, suggesting that there is also uptake from the basal side.⁸¹

Gross tissue distribution of PS ODNs is independent of sequence. Plasma clearance rates are largely species independent in rat, rabbit, dog, and monkey and has been estimated to be between 1 and 3 ml/min/kg.^{82,83} DeLong et al. (1997) compared the pharmacokinetics and tissue distribution of PS. phosphorodithioate (PS₂), MP ODN analogs, which were 15-nt ODN complimentary to the AUG region of K-ras.⁸⁴ These ODNs were injected intravenously as a single dose used in nude mice bearing a K-ras-dependent human pancreatic tumor. There was a rapid distribution phase with $t_{\frac{1}{2}} \alpha$ values of 1 min and an elimination phase with average $t_{\frac{1}{2}\beta}$ by values of 24 or 35 minutes. Volumes of distribution (V_d) were 3.2, 4.8, and 6.3 mL for PS2, MP, and PS, respectively, in comparison to 3.6 mL for sucrose, a fluid-phase marker. In general agreement with previous studies, relative tissue drug levels obtained at 1 and 24 h after administration were kidney > liver > spleen > tumor > muscle. Total kidney and liver ODN accumulation was approximately 7 to 15% of the initial dose, with tumor accumulating 2 to 3%. Intact compound was recovered from all tissues, including tumor. Importantly, integrity of the ODNs ranged from 73% in blood to 43 to 46% in kidney and liver, which appear to be the primary sites of metabolism.

In humans, studies have been performed on patients with leukemia and acquired immunodeficiency syndrome (AIDS). In six HIV patients, after 2-hr intravenous infusions (0.1 mg/kg) of PS, plasma disappearance curves could be described by the sum of two exponential, with half-life values of 0.18 ± 0.04 and 26.71 ± 1.67 hr. Urinary excretion represented the major pathway of elimination, with ~50% of the administered dose excreted within 24 hr and ~70% eliminated over 96 hr after dosing. Intact and degraded material was found in the urine. Of interest, the half lives of the ODNs were shorter than those observed in experimental animals.

19.9 CELLULAR UPTAKE OF OLIGONUCLEOTIDES

Cellular uptake of ODNs appears to be an active process dependent on cell type, time, concentration, energy, temperature, saturable as well as sequence and type of ODNs.^{85–87} A number of laboratories have examined ODN uptake using either native phosphodiester (PO), methylphosphonate (MP),

or phosphorothioate (PS) ODNs. Cellular uptake of ODNs is highly variable and dependent on cell type and cell cycle.⁸⁸ MP ODNs are uncharged molecules that have been reported to enter cells via passive diffusion,⁸⁹ although the rate of diffusion across the lipid bilayer membrane is extremely slow.⁹⁰ In contrast, PO and PS ODNs are polyanionic molecules, which make it difficult for them to passively diffuse across cell membranes. The uptake mechanism appears to be at least partially concentration dependent and that below a concentration of 1 μ M, uptake of PS ODNs is predominantly via a receptorlike mechanism, while at higher concentrations a fluid-phase endocytosis mechanism appears to predominate. There are reports suggesting the presence of ODNs within clathrin-coated pits on the cytoplasmic membrane upon cellular internalization.

Several strategies have been developed to enhance the cellular uptake of these ODNs to target cells. ODN form complexes with cationic liposomes, which can condense and impart an overall positive charge that facilitates attachment to the cell membrane while the lipid tails enhance subsequent passage through the lipid bilayer of the cell membrane.^{91,92} Cationic liposomes/ ODN complexes are taken up by endocytosis and ODNs are released from the liposomes into the cytoplasm due to the protonation of the lipids.⁹³ Once free in the cytoplasm ODNs diffuse into the nucleus. A specific antibody or ligand is conjugated to carrier molecules to allow specific delivery of ODNs.

The backbone of ODNs are often modified to enhance their cellular uptake. The strategy most frequently used has been to conjugate the oligomer to a ligand specific for a receptor reside on the cell type of interest. For example, ODNs have been conjugated to folate, mannose, asialoglycoproteins, and tumor-specific antibodies to target hematopoietic, pulmonary alveolar, hepatocytes, and a variety of tumor cells. While such modifications have reported to be effective in certain controlled circumstances, the general applicability of this approach remains to be determined. However, to make this approach successful, several obstacles need to be overcome including the (i) inefficient dissociation of ODNs from the carrier after endocytosis, (ii) uptake by reticuloendothelial (RES) and other non-target cells.

Microinjection of fluorescent-labeled ODNs into the cytoplasm of cells has been shown to result in their rapid accumulation in the nucleus. In contrast, when cells were incubated with these fluorescent-labeled ODNs, these labeled molecules were seen to accumulate mainly in the endosomal/lysosomal vacuoles and small percentage in the perinuclear membranes, but little inside the nucleus. This indicates that the ODN release in the cytoplasm and their translocation to the nucleus is an inefficient process.

To enhance the cellular uptake and nuclear translocation of ODNs, Nori et al. used TAT conjugated HPMA copolymer for ODN delivery and demonstrated very high concentration of ODNs in the nucleus.¹⁹ TAT is a peptide derived from HIV-1 tat protein that can mediate cytoplasmic transport of other compounds to which they are thethered, whereas HPMA is a nontoxic copolymer.

19.10 DELIVERY STRATEGIES OF OLIGONUCLEOTIDES

To enhance stability, control the pharmacokinetic profiles and to facilitate site-specific delivery of ODNs, both macromolecular conjugates and particulate systems are being developed. ODNs can be linked directly to a carrier protein or targeting ligand via a covalent bond or noncovalently via polycation-carrier conjugates. The choice of a carrier protein is dependent on its known ability to bind to specific cell membrane receptors and accumulate in the cell via endocytosis.

As a first step towards the use of receptor-mediated ODN delivery to different liver *cells*, we determined the disposition characteristics of ³⁵S-labeled ODNs and their glycosylated PLL complexes in mice in relation to their physicochemical properties. Both hepatocytes and Kupffer cells possess receptors on their plasma membranes that specifically bind and internalize D-galactose-containing materials in size-dependent manner: small particles are efficiently taken up by hepatocytes, whereas large particles are taken up by Kupffer cells.⁹⁴ Complex formation with galactosylated poly(L-lysine) (Gal-PLL enhanced the hepatic uptake of ODNs (Figure 19.8). Although the uptake of PS/Gal-PLL complexes by PC was significantly higher than that of naked PS, the difference between their intracellular distributions was only moderate (Figure 19.9). The hepatic uptake of PS/Gal-PLL was partially inhibited by prior intravenous administration of excess Gal-BSA, suggesting that the complexes were taken up by the hepatocytes via galactose receptor-mediated endocytosis. Due to the negative zeta potential (-30 to -40 mV) and



FIGURE 19.9 Hepatic cellular localization of ³⁵S-ODNs administered intravenously alone or after complex formation with Gal-PLL and Man-PLL in mice. PC, parenchymal cells; NPC, nonparenchymal cells. Although Gal-PLL enhanced the uptake of PS ODNs by the hepatocytes, significant amount was non-specifically taken up by the liver PC and NPC.

wide particle size distribution $(150 \pm 70 \text{ nm})$, a part of the complexes is likely to be recognized by the galactose receptor of Kupffer cells, by scavenger receptors of the endothelial cells and Kupffer cells as polyanions⁹⁵ and/or being phagocytosed by Kupffer cells. Moreover, sulfur atoms present in the PS ODN molecules also influences the nonspecific hepatic uptake of its complexes. In conclusion, hepatic uptake of ODNs/glycosylated PLL is greatly influenced by the particle size, zeta potential, sugar substitution level, molecular weights of both polycations and ODNs, and types of ODNs.^{10,96} To minimize nonspecific ionic interaction with plasma proteins and cytotoxicity, poly(ethylene glycol) (PEG) can be used as spacer between polycations and the targeting ligands.

To avoid the use of polycations by direct conjugation with the ligands, Rajur et al. (1997) covalently conjugated ODNs to asialoglycoprotein by a stable disulfide linkage.⁹⁷ ODNs were also covalently conjugated to carbohydrate cluster for specific delivery to the hepatocytes.⁹⁸ This conjugation strategy can also improve the stability of ODNs by introducing ligands and appropriate cationic tethers, which can interfere with interactions between nucleotlytic enzymes and ODNs. Consequently, this conjugation approach will have significant influence on the bioactivity of ODNs.

Enhanced cellular uptake of morpholino oligomers by epidermal growth factor (EGF) receptor-mediated endocytosis in lung cancer cell line NCI-H125 has been demonstrated.⁹⁹ Therefore, receptor-mediated endocytosis offers the potential to deliver ODNs to different target cells. The particle size of ODN delivery systems must be very small so that they can easily pass through the sinusoidal gaps for efficient delivery to hepatocytes and hepatic stellate cells (HSC). Moreover, the formulated ODN particles should not carry excess positive charge on their surface otherwise they will be taken up by Kupffer cells before reaching hepatocytes and HSC.⁹⁶

19.10.1 CONJUGATION OF LIPOPHILIC MOLECULES

ODNs are hydrophilic molecules by virtue of their phosphate and sugar backbone. The hydrophilic character and anionic backbone of the ODNs reduces their uptake by the cells. Therefore, various lipophilic molecules have been conjugated to ODNs.¹⁰⁰ Among them, cholesterol is perhaps the best characterized. Biophysical and pharmacokinetic properties of 20-mer PS ODNs and its cholesterol analogs have been evaluated. As shown in Figure 19.10, cholesterol conjugation to PS ODNs resulted in increased retention in plasma as well as accumulation in various liver cell types, with up to five-fold increase in the uptake by Kupffer cells.^{101,102} Cholesterol conjugated PS ODNs (ISIS-8005) showed greater affinity to serum proteins than free PS ODNs (ISIS-3082). This difference in serum protein binding is responsible for the difference in pharmacokinetics. The concentration in liver was correlated with the therapeutic effect of cholesterol conjugated PS ODNs as measured by ICAM-1 mRNA levels in mouse liver *in vivo*, whereas free PS ODNs showed little effect.¹⁰³ Moreover, the presence



Comparison of hepatic cellular distribution of 20 mer PS ODNs with and without lipid conjugation

	Contribution to liver uptake (% of total)		
Cell type	ISIS-3082	ISIS-9388	ISIS-9388/LacLDL
Kupffer cells	$\textbf{8.3}\pm\textbf{3.2}$	$\textbf{14.5} \pm \textbf{3.1}$	$\textbf{43.9} \pm \textbf{5.4}$
Endothelial cells	$\textbf{62.5} \pm \textbf{6.2}$	$\textbf{55.9} \pm \textbf{7.2}$	$\textbf{39.4} \pm \textbf{3.8}$
Parenchymal cells	29.1 ± 6.3	$\textbf{29.6} \pm \textbf{8.1}$	$\textbf{16.7} \pm \textbf{6.8}$

FIGURE 19.10 Sequence and hepatic cellular distribution of 20-mer phosphorothioate oligonucleotides (PS ODNs) with and without lipid conjugation. Reproduced with some modifications from Bijsterbosch, M. K. et al. (2001) *Biochem. Pharmacol.*, **62:** 627–633.

of cholesterol moiety at the 3' end of an ODN did not affect hybridization with target sequences.

However, this 5'-cholesterol conjugate of PS ODNs (ISIS-8005) did not change significantly the stability of ODNs, whereas the 3'-cholesterol conjugate (ISIS-9388) of ISIS-3082 was much more stable.¹⁰² This is because the 3'-hydroxyl group, which is involved in the nucleophilic attack of the adjacent phosphate bond when the exonuclease enzyme makes a complex with the nucleic acid, is unavailable in ISIS-9388.

Many other studies^{17,104} have shown similar effects in increasing the cellular uptake of ODNs when cholesterol was conjugated with ODNs. Cellular uptake of 3'-cholesterol-conjugated ODNs has been examined with a real-time confocal laser microscopy. Cytosolic uptake of cholesterol conjugate was five times as rapid as that of PS ODNs and nuclear uptake of cholesterol conjugate was twice as fast as that of unmodified PS ODN.

The effects of conjugating cholesterol to either or both ends of a phosphorothioate ODN were compared in terms of cellular uptake and antisense efficacy against the p75 nerve growth factor receptor (p75) in differentiated PC12 cells.¹⁰⁵ The addition of a single cholesteryl group to the 5' end significantly increased cellular uptake and improved p75 mRNA down-regulation compared with the unmodified ODN. While the 3'-choloesterol analogue was still active, bis-cholesteryl (5'- and 3'-) conjugated ODN was even more potent. LeDoan et al. (1999)¹⁰⁶ reported on the interactions of phosphodiester ODNs linked to the cholesterol group at internal position. The conjugates were assessed for their capacity to bind, penetrate, and partition in the cytoplasmic compartment of murine macrophages and showed similar effects. In addition to effects on cellular uptake, cholesterol modulates ODN-mRNA hybrid stability via hydrophobic interactions.¹⁰⁷ An increase in the $T_{\rm m}$ of up to 13.3 °C was observed. Stabilization of triplexes by up to 30 °C due to intercholesteryl interaction using 5',3'-bis-cholesterol-containing ODNs was also observed.

However, the mechanism of cellular uptake of cholesterol-conjugated ODNs has not been clearly established, although a receptor-mediated process involving lipoproteins has been implicated. The lipophilicity of the hydrophobic steroid skeleton of cholesterol may be optimum to enhance cellular association; cholesterol conjugates may form micellar structures that facilitate the cellular uptake.

19.10.2 CONJUGATION OF VITAMINS

Cells and tissues have specific transport systems for vitamins. Conjugation of antisense ODNs to vitamins is expected to improve their transport into cells. A number of vitamins, including folic acid, biotin, retinoic acid, and vitamin E have been investigated.¹⁰⁸ Among them, folic acid is paid most extensive attention. Folate receptor is overexpressed on many cancer cell surfaces, but is highly restricted in most normal tissues. A conjugate was prepared by directly coupling folic acid to the 3' terminus of an anti-*c*-fos ODN. Its cellular uptake by FD2008 cells that overexpress folate receptors, was increased by eight-fold, but did not increase in CHO cells that do not express folate receptors.¹⁰⁸ The inhibitory effect of the ODNs on the growth of FD2008 cells was significantly increased when ODNs were conjugated to folic acid conjugated compared to unmodified ODNs.

19.10.3 PEGYLATION OF OLIGONUCLEOTIDES

As discussed in Chapter 5, poly(ethylene glycol) (PEG) plan an important role in the pharmacokinetic profiles of therapeutic proteins. Manoharan et al. showed that when an ODN that targets human ICAM-1 has been conjugated to a series of PEG esters of average molecular weight 550, 2200, and 5000, the cellular permeation of ODNs in vitro can be interfered.¹⁰² However, the effect of the different structures of high-molecular-weight PEG chains on the biological properties of the conjugated antisense ODNs have been investigated by Bonora et al. (1997).¹⁰⁹ Two different conjugates of an anti-HIV 12-mer ODN have been tested for antisense activity in MT-4 cells. Only the ODN conjugated to the linear monomethoxy PEG showed anti-HIV activity. A 20-mer ODN targeting mouse β -globin mRNA has been conjugate at the 5'-terminus to *bis*-aminoalkyl PEG. At 15 μ M the conjugate selectively inhibited Hb synthesis in cultured Friend murine erythroleukemia cells by 95%. Bonora et al. (1997)¹⁰⁹ conjugated PEG to ODNs at the 5', 3', or both 5' and 3' termini. The number and attachment

sites (3'-terminal, 5'-terminal, and internal positions) of coupled ethylene glycol units had great influence on the hydrophobicity and electrophoretic mobility of the conjugates. PEGylation had little effect on the hybridization behavior of ODNs. Conjugates with PEG coupled to both 3'- and 5'- terminal positions showed a more than 10-fold increase in exonuclease stability of the ODNs.

19.10.4 MULTIVALENT CARBOHYDRATE CLUSTERS

In vivo study of distribution of a PS ODN within rat liver after intravenous administration has revealed an important aspect of distribution of antisense ODNs: distribution of ODNs to a tissue does not mean that ODNs have been localized to target cells within that tissue. Graham et al. (1998)¹¹⁰ have shown that after a 10 mg/kg dose of phosphorothioate ODNs, nonparenchymal (i.e., Kupffer and endothelial) cells contained approximately 80% of the total organ dose, equivalently distributed between the two cell types. Only 20% of the ODNs was associated with hepatocytes. Nonparenchymal cells contained abundant nuclear, cytosolic, and membrane drug levels over a wide dose range, while hepatocytes had no detectable nuclear association. These results suggest that efficient methods must be devised to deliver ODNs to hepatocytes.

Carbohydrate cluster conjugates may offer a solution to this problem. When a MP ODN was conjugated to the triantetennary, N-acetylgalactosamine neoglycopeptide, Tyr-Glu-Glu-(aminohexyl GalNAc)₃, YEE(ahGal-NAc)₃, there were 20–40 fold enhancement in the uptake of the ODNs by HepG2 cells.¹¹¹ These carbohydrate clusters are known to bind to Gal/GalNAc receptor sites on hepatocytes with high affinity ($K_d \sim 7$ nM). Similarly, when a PS oligomer was conjugated to this glycotripeptide, there was sequence-specific suppression of the integrated HBV viral expression in hepatoma cells (greater than 90% inhibition) in a dose-dependent concentration range of 1 to 20 μ M of ODNs.¹¹²

Galactose is the ligand for the asialoglycoprotein receptor, which is expressed on the surface of liver parenchymal cells. Biessen et al. (1999, 2000)^{113,114} constructed four molecules of galactoses on a oligolysine (N2-(N2-(N2,N6-bis[N-[p-(β -D-galactopyranosyloxy)-anilino]thiocarbamyl]-Llysyl)-N6-(N-[p-(β -D-galactopyranosyloxy)-anilino]thiocarbamyl]-L-lysyl)-N6-(N-[p-(β -D-galactopyranosyloxy)-anilino]thiocarbamyl]-L-lysyl)-N6-(N-[p-(β -D-galactopyranosyloxy)-anilino]thiocarbamyl])-L-lysine, (L3G4)), and attached it to a 20-mer PO ODN 3'-capped with an amine. The ligand has a K_d of 6.5 nM. This L3G4-conjugated ODN was far more efficiently bound to and taken up by parenchymal liver cells than underivatized ODN. Studies in rats showed that hepatic uptake was greatly enhanced from 19% for unconjugated ODN to 77% of the injected does after glycoconjugation. Accumulation into liver parenchymal cells was increased by 60-fold.

19.10.5 CONJUGATION OF POLYAMINES, CATIONIC GROUPS, AND POLYPEPTIDES

Polyamine-conjugated ODNs form amphipathic molecules and reduce the net negative charge on ODNs. As these modified ODNs may serve as ligands for polyamine receptors present on certain cells, polyamine conjugation may improve the antisense activity of ODNs. Since the monoalkylamines and at least some of the amino groups of the polyamines are protonated at the physiological conditions, they can also improve the hybridization rates of antisense ODNs to the target RNA. Corey (1995) demonstrated 48,000-fold acceleration of hybridization by ODNs conjugated to cationic peptides derived from lysines. Cationic polypeptides (polylysine, polyornithine, polyhistidine, and polyarginine), due to their positive charges, when conjugated with ODNs, which are negatively charged, can enhance hybridization of ODNs.

A fusogenic peptide derived form the influenza hemagglutinin envelope protein has been conjugated to antisense ODNs. This peptide changes conformation at acidic pH and destabilized the endosomal membrane resulting in an increased cytoplasmic delivery of the antisense drug. A similar fusogenic peptide conjugated to an antisense ODNs via a disulfide or thioether bond resulted in 5- to 10-fold improvement of the anti HIV activity on de novo infected CEM-SS lymphocytes in serum free media. However, no sequence specificity was observed and the fusogenic peptide possessed some antiviral activities on its own.

19.11 CONCLUDING REMARKS

Oligonucleotide-based approaches have great potentials in treating many diseases by inbihiting gene expression in a sequence specific way. While antisense ODNs are used for inhibiting translation of mRNA into proteins, TFOs can be used for inbihiting gene expression via site-specific gene targeting. There have been major advances in the development of various backbone modified ODNs and macromolecular and particulate delivery systems, which can be used for transporting these molecules to specific disease targets. However, development of successful ODN-based therapeutics requires a thorough understanding of the relationship among their backbone structures, pharmacokinetic proifiles, cellular uptake mechanisms and therapeutic activity of ODNs with or without a delivery system.

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20 Artificial Nucleic Acid Chaperones

Atsushi Maruyama

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References

20.1 INTRODUCTION

Association, dissociation, and strand exchange of nucleic acid hybrids are pivotal processes in maintenance of living systems. Various types of nucleic acid acting proteins, such as helicases, recombinases and nucleic acid chaperones, are involved in the regulation of structural transition of nucleic acids. An artificial agent that is capable of manipulating nucleic acid hybridization would be beneficial in the field of DNA nanobiotechnology. These agents would be important to design various types of DNA-based devices for DNA nanomachines,^{1,2} nanoassembly,^{3,4} and molecular computing^{5,6} and to refine analytical and genotyping methods for basic and diagnostic studies. Also, such agents are useful to elaborate DNA medicines that target a particular gene or its transcript in order to inspect and engineer gene expression.

In the cell, newly synthesized proteins can either begin to fold while still attached to the ribosome or after they are released. Either way, they quickly collapse to an intermediate to the native state. If the final folding steps are slow, the intermediate whose hydrophobic residues may be more exposed to solvent than the native state, can aggregate non-specifically into disordered amorphous aggregates. Depending on the specific protein, sometimes ordered or fibrillar amyloid-like aggregates may be formed. Native proteins can be further destabilized or unfolded by heat or other types of stress contributing to the pool of aggregation-prone intermediates. Molecular chaperones effectively promote folding. In this chapter, I discuss the possible use of cationic combshaped copolymer as an artificial chaperone for nucleic acid folding.

20.2 NUCLEIC ACID CHAPERONES

Molecular chaperones are abundant proteins in cells that interact transiently with incompletely or misfolded proteins to facilitate correct folding, assembly, or translocation across intracellular membranes. The term "molecular chaperone" was first used by Laskey et al.⁷ to describe nucleoplasmin, an acidic nuclear protein required for assembly of nucleosomes from DNA and histones. Rapid mixing of separated DNA and histones at physiological ionic strength results in a precipitate of heterogeneous aggregates between them (Figure 20.1). Nucleoplasmin does not bind to DNA or to nucleosomes, but interacts with histones in such a way that it shields their positive charges. Nucleoplasmin promotes histone–histone assembling by reducing electrostatic repulsion between them, and minimizes the formation of insoluble nonspecific aggregates by competing with the electrostatic attraction between the histones and DNA (Figure 20.1).⁸ Nucleoplasmin is required only for assembly and itself does not form part of the nucleosomes, nor does it carry any steric information specific for nucleosome assembly.

Molecular chaperones do not form part of the final structure nor do they necessarily possess steric information specifying assembly. Proteins that fall within the proposed class of molecular chaperones have been described from viral, animal, and plant sources. Especially, a number of chaperone proteins



FIGURE 20.1 Chaperoning function of nucleoplasmin in nucleosome assembling.



FIGURE 20.2 Denature and reassociation of nucleic acids.

that support folding of polypeptide chains and refolding from denatured structure was identified.

Beside the molecular chaperones for polypeptide folding, a class of proteins that ensure hybridization of nucleic acid has been described.⁹ When a solution of a duplex DNA is heated, the double helical structure undergoes transition to single stranded form (Figure 20.2). The half melting temperature is defined as melting temperatures (T_m) of the double stranded DNA. The resulting single stranded DNAs hardly refold into the original double helical form upon rapid cooling of the solution. These DNAs incompletely reassembled into thermodynamically mesostable structures, such as intramolecular folding structures, with partial base pairing. These mesostable structures rather than the most optimal structure preferentially form owing to kinetic advantages. The DNAs with the suboptimal structures hardly refold into the perfect one because of a high energy barrier for dissociation and reassociation of base pairs.

While the thermodynamically most optimal structure can be obtained by annealing, heating followed by slow cooling of the DNA solution (Figure 20.2), in living cells nucleic acid chaperones play a role to reassemble correctly DNAs and RNAs at a physiological temperature. The nucleic acid chaperones interact with nucleic acids to reduce the energy barrier associated with breakage and reassociation of nucleic acid base pairs and catalyze structural transition of nucleic acids into the thermodynamically most stable structure, usually hybrid with the highest number of base pairs (Figure 20.3).

Retroviral nucleocapsid proteins are the class of nucleic acid chaperones.¹⁰ The proteins are small, basic and have one or two Zn finger motifs (Figure 20.4). The proteins accelerate nucleic acid hybridization and strand exchange, which are considered critical during the initial stages of reverse transcription. Nucleic acid chaperoning activity of the proteins likely involves helix destabilizing properties.¹¹ However, the properties may not be favorable for their application to DNA nanobiotechnology, because stable and robust helical structures are required. For example, stable duplex is needed for DNA analytical methods with probe/primer hybridization process and molecular



FIGURE 20.3 Thermodynamic role of nucleic acid chaperones.



FIGURE 20.4 HIV1 nucleocapsid protein, NCp7. Cationic amino acid residues are written with bold face.

construction of devices for nanoassembly. Furthermore, instability and insufficient availability of naturally occurring nucleic acid chaperones may critically limit their usage in nanotechnology and biotechnology employing nucleic acids.

20.3 DESIGN OF ARTIFICIAL MATERIALS FOR ENHANCING NUCLEIC ACID HYBRIDIZATION

Due to inadequacy of native nucleic acid chaperones for application to DNA nanotechnology and biotechnology processes, we are interested in designing artificial chaperones. Our interest is to design materials that can enhance internucleotide recognition and promote hybridization. Nucleic acid hybridization is driven by various intermolecular forces such as hydrogen bonding as well as hydrophobic and stacking interactions between nucleotides. On the other hand, hybridization is hampered by the electrostatic repulsion between nucleotides, because charge density of anionic groups increases with hybridization. Indeed, it is well established that stability of nucleotide hybrids is higher in a medium with high ionic strength, a condition which alleviates electrostatic repulsion.^{12,13} Thus, it may be possible to enhance hybridization by shielding the electrostatic repulsion. One of the strategies to increase hybrid stability by minimizing electrostatic repulsion was a design of noncharged nucleic acid analogue like peptide nucleic acids (PNA).^{14,15} However, a method to enhance hybridization of native nucleic acids should have wider range of applications.



FIGURE 20.5 Design of cationic comb-type copolymers as nucleic acid chaperoneinspired materials.

We employed cationic polymers to shield the electrostatic repulsion between the nucleic acids. Cationic polymers, polycations, interact with anionic polymers, polyanions, to form stable complexes where electric potentials of polyions counteract each other. The complexes are referred to as the interpolyelectrolyte complex (IPEC). IPEC formation occurs even at very low concentrations of both polyions, so that promotion of hybridization can be achieved with a minimum amount of polycations. We have synthesized a cationic comb-type copolymer poly(L-Lysine)-g-Dextran (PLL-g-Dex), consisting of a cationic poly(L-lysine) backbone and hydrophilic side chains of dextran (Figure 20.5).^{16,17} The dextran graft chains of PLL-g-Dex guarantee solubilization of IPECs between polylysine and DNA. Furthermore, the graft chains of dextran play a role to prevent DNAs in the IPEC from induced structural changes that might be unfavorable for correct hybridization.

The ability of PLL-g-Dex to promote DNA hybridization was examined by measuring $T_{\rm m}$ of DNA hybrids.^{18,19} Effect of the copolymer on the melting properties of poly(dA):2 poly(dT) triple helical DNA was shown in Figure 20.6. Poly(dA):2 poly(dT) melted with bimodal transition. The first transition at the lower temperature, 37° C, is transition from the triple helix to poly(dA): poly(dT) double helix, and that at the higher temperature, 72° C, is melting of poly(dA): poly(dT) double helix to single-stranded DNAs. In the presence of PLL-g-Dex, poly(dA): 2 poly(dT) triplex showed one-step melting at about 90°C. Electrostatically small excess amount of PLL-g-Dex increased $T_{\rm m}$ of poly(dA): 2 poly(dT) triple helix and poly(dA): poly(dT) double helix



FIGURE 20.6 Effect of a cationic comb-type copolymer, poly(L-lysine)-graft-dextran (PLL-g-Dex) on melting and reassociation of poly(dA)·2poly(dT) triple helical DNA.

nearly 50°C and 20°C, respectively, indicating strong stabilizing activity of PLL-g-Dex in nucleic acid hybridization. In spite of the strong stabilizing activity of PLL-g-Dex and thereby seemingly strong interaction between them, reassociation of hybrids in the cooling process was confirmed. Our thermodynamic and kinetic studies revealed that PLL-g-Dex increased the stability of triple helical DNA by 100 holds and increase its association rate by 50 fold.^{20,21} In other words, interaction between the copolymer and DNA does not retard but rather accelerate supramolecular formation between nucleic acids. Stabilizing activity of the copolymer toward triple helical DNAs were shown to be effective on a real sequence²² and useful for sequence selective inhibition of DNA/protein interactions.²³

20.4 CHARACTERIZATION OF CATIONIC COMB-TYPE COPOLYMER AS A NUCLEIC ACID CHAPERONE

The most striking point in the copolymers activities is that PLL-g-Dex increases the association rate in nucleic acid hybridization while it stabilizes the hybrids. Thus, the copolymer may have the stabilizing effect not only on matured hybrids but also on the transition complexes in hybridization process. PLL-g-Dex is likely to decrease the activation free energy accompanying with the hybridization process. To inspect this possibility, effect of the copolymer on strand exchange reactions of DNAs was examined. DNA strand exchange reactions are reactions between double stranded DNAs and its homologous single stranded DNAs (Figure 20.7, left panel). For the strand exchange reaction to occur breakage and reassociation of base pairs should be kinetically facilitated. Since composition of the reaction mixture is not changed before and after the reaction, the reaction does not involve free energy change of the system. Hence, we can directly assess the effect of PLL-g-Dex on the transition state of the reaction. If PLL-g-Dex accelerates the strand exchange reaction, we



FIGURE 20.7 FRET detection of DNA strand exchange reaction (left panel) and accelerating effect of PLL-*g*-Dex (right panel).

can conclude that they reduce the activation free energy between breakage and reassociation of base pairs and act as a nucleic acid chaperone.

The strand exchange reaction between 20 base pair (bp) double-stranded DNAs and homologous single-stranded DNAs were examined in the absence or presence of PLL-g-Dex.^{24,25} The strand exchange reaction was monitored by fluorescence resonance energy transfer (FRET) method employing double-stranded DNA labeled with both energy donor and acceptor fluorophores. Progress in the exchange reaction was detected by recovery of the donor fluorescence which had been quenched by FRET. In the absence of the copolymer the exchange reaction hardly occurred. One hour incubation at physiological conditions resulted in the strand exchange of less than 10% (Figure 20.7, right panel). When PLL-g-Dex was added in the mixture, the reaction rapidly proceeded and achieved to an equilibrium within a few minutes (Figure 20.7, right panel). PLL-g-Dex accelerates the strand exchange reaction by more than four orders. We compared the activity of PLL-g-Dex with a NC protein, NCp7, a naturally-occurring nucleic acid chaperone. The protein, however, only shows slight effects (0.2% of the intrinsic activity of PLL-g-Dex) in strand exchange acceleration under our experimental conditions. The binding affinity of NCp7 is sequence-selective, and this might be unfavorable for its uses in nucleic acid biotechnology and nanotechnology.

It is unique that PLL-g-Dex accelerates strand exchange reaction while stabilizing double stranded DNAs. This property of PLL-g-Dex makes a clear contrast with NC proteins that destabilizes DNA hybrids. The mechanism involved in the chaperoning activity of PLL-g-Dex is not fully understood. The ionic interaction between PLL-g-Dex and DNAs is, however, essential because the activity of PLL-g-Dex is weaken as ionic strength of the media increases. PLL-g-Dex likely stabilizes the transition complexes of the exchange reaction by shielding ionic repulsion between the DNA strands.²⁵

20.5 DNA ANALYSIS USING CHAPERONING ACTIVITY OF PLL-g-Dex

Genetic diagnoses, such as single nucleotide polymorphism (SNP) typing, allow elucidation of gene-based physiological differences, such as susceptibility to diseases and response to drugs, among individuals. Many detection technologies, including allele-specific hybridization, allele-specific primer extension and oligonucleotide ligation, are being used to discriminate SNP alleles.^{26,27} These methods still have many unsolved practical issues.^{26–28} In general, they require adequate and specific hybridization of primer or probe DNAs with target DNAs. This frequently needs optimization of the probe/primer structures and operating conditions. In nature, highly homology-sensitive hybridization is assisted by a nucleic acid chaperone that reduces the energy barrier associated with breakage and reassociation of nucleic base pairs. Thus, we challenged to use cationic comb-type copolymers producing high nucleic acid chaperone activities in single base mismatch analysis.

We have focused on the strand exchange reactions (SERs) between doublestranded (ds) DNA probes and single-stranded (ss) target DNA as a format for detection of single-base mismatches (Figure 20.8). As shown in Figure 20.8, the SER is initiated by nucleation of the ss target to the partially unwound duplex, followed by rapid branch migration.²⁹ Both nucleation (Figure 20.8, right route) and branch migration (Figure 20.8, central route) of the heteroduplex are likely to be strongly retarded when the target single-stranded DNA involves a single-base mismatch. Hence, it seems possible to discriminate the existence of a single-base mismatch by SER rates regardless of the position of



FIGURE 20.8 Strand exchange of a double-stranded DNA with a single-stranded target with or without single-base mismatch.



FIGURE 20.9 Effect of single-base mismatch on the polycation accelerated strand exchange.

mismatches. A drawback of the assay could be an extremely slow rate for the overall SER. Previously, partial duplex probes have been used to facilitate SER, and discrimination of mismatches near the central portion of the probes has been demonstrated.^{30,31} However, mismatch discrimination near probe ends is considered difficult, because the nucleation process is driven by the single-stranded portion of the partial duplex probes. Furthermore, like other hybridization-based detection technologies using probes or primers, hybridization of the probe should be strongly impeded by intramolecular folding structures of target DNAs.

The chaperoning and stabilizing activity of PLL-g-Dex prompted us to exploit their activities in the strand exchange assay (polycation accelerated strand exchange, PASE) for single-base mismatch detection, because both the chaperoning and stabilizing activities of PLL-g-Dex are favorable for the assay. First, we assessed the effect of single-base mismatches on strand exchange kinetics by using a FRET assay. As aforementioned, the strand exchange with perfectly homologous single-stranded DNA was promoted in the presence of PLL-g-Dex (Figure 20.9). However, a singlebase mismatch in the single stranded target significantly retarded the reaction even in the presence of PLL-g-Dex.³² The result suggested that discrimination between full matched and single-base mismatched sequences was achieved within a few minutes by a simple incubation of a double-stranded probe with target single-stranded DNAs. The PASE was then challenged with diverse mismatches in various sequences (Figure 20.10).³³ Regardless of mutation, single-stranded targets containing single-base mutations could be distinguished quickly from fully-matched DNAs. The signal intensity ratios from fullymatched sequences relative to mismatched sequences in Figure 20.10 were more than 10 at incubation periods ranging from 1 to 10 min. Target sequences in Figure 20.10 have a mutation that breaks an A:T base pair, causing a slight change in the thermodynamic stability of the duplexes. In particular, the



FIGURE 20.10 PASE discrimination of 20-mer DNA with or without single-base mismatch causing disruption of A-T base pair. dsDNA probe; F2: 5'-ATGGTGAG-CAAGGGCGAGGA-3'-FITC; T2: 3'-TACCACTCGTTCCCGCTCCT-5'-TAMRA; ssDNA Target; M2: 3'-TACCACTCGTXCCCGCTCCT-5'; X = T (full match), G, A, C (mismatch).



FIGURE 20.11 Effect of single-base mismatch on melting temperatures of 20 bp double-stranded DNAs.

change, $\Delta T_{\rm m}$, in melting point between the fully matched and the A:G mismatched duplexes is only 5°C (Figure 20.11). Furthermore, the duplex melting profiles largely overlapped that of the fully-matched duplex, as shown in Figure 20.11. These results indicate that the different sequences are hardly discriminated by thermal analyses or regular probe hybridization using single-stranded DNA as a probe. Nevertheless, these mismatched sequences were clearly discriminated from the fully-matched sequences using the PASE assay.



FIGURE 20.12 PASE discrimination of 20-mer DNA with or without single-base mismatch causing disruption of A-T base pair at varying position. dsDNA probe; F2: 5'-ATGGTGAGCAAGGGCGAGGA-3'-FITC; T2: 3'-TACCACTCGTTCCCGCTC CT-5'-TAMRA; ssDNA Target; M2: 3'-TACCACTCGTT₁₀CCCGCT₄CCT₁-5'.



FIGURE 20.13 Effect of single-base mismatch on melting temperatures of 20 bp double-stranded DNAs.

In general, mismatches at the duplex end have less effect than those at the duplex center, and are difficult to discriminate by using hybridizationbased methods. We then explored PASE with single-stranded targets having mismatches at various locations. In Figure 20.12, the results of PASE with single-stranded targets causing an A:G base mismatch at various locations are shown. As seen from the melting profiles for these duplexes in Figure 20.13, differences in melting profiles between the fully-matched and the single-base mismatched duplexes became less apparent when the mismatched base pair was near the duplex end. A:G mismatches at the duplex end only produced a $\Delta T_m \approx 1^{\circ}$ C. Despite this subtle influence of the terminal mismatch on the thermodynamic stability, PASE differentiates these sequences (Figure 20.12).

Another feature of PASE assay is that PASE assays have a wide window for operating temperature and is favorable for simultaneous analyses of multiple sequences. Furthermore, mismatch discrimination is attained using incubation temperatures ranging from 37°C to 25°C. Accurate temperature control has been a prerequisite for any DNA analytical methods because proper hybridization of probe or primer sequences to a target sequence was pivotal.^{34,35} In contrast to these assays, PASE reduces or omits time-and resource-consuming steps, such as probe design and conditional tuning, often required for high-throughput SNP typing assays.

20.6 CONCLUDING REMARKS

The cationic comb-type copolymers having hydrophilic side chains exhibited strong stabilization effect on double-helical and triple-helical DNAs. The copolymers also showed accelerating effect on both hybrid formation and strand exchange reactions. The copolymer showed unique chaperoning activity toward nucleic acids. Unlike natural occurring nucleic acid chaperones the copolymer did not destabilize hybrid structures. The mechanism for decreasing activation free energy associated with the chaperoning activity of PLL-g-Dex is not fully solved. However, the unique activity of PLL-g-Dex may open a novel strategy for nanobiotechnology employing nucleic acids and its molecular recognition. Indeed, the copolymers were shown to be useful for DNA analysis. Use of the copolymer with enzyme-assisted technologies would also be exciting because this mimics roles of nucleic acid chaperone in living system.

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21 Basic Components of Plasmid-Based Gene Expression Systems

Minhyung Lee and Sung Wan Kim

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21.1 INTRODUCTION

Plasmids have been widely used for nonviral gene therapy to deliver genetic information to target cells. A plasmid has various advantages as a carrier of



FIGURE 21.1 Map of a mammalian expression plasmid. A therapeutic plasmid should contain DNA elements for gene expression in mammalian cells. The DNA elements are enhancer, promoter, intron, therapeutic gene and polyadenylation signal. In addition, a bacterial replication origin (Ori) should be included for propagation in bacteria. Antibiotic resistance gene is for bacterial selection.

genetic information. First, a plasmid can be amplified in bacteria easily in a large quantity. Second, a desired plasmid can easily be selected using antibiotic resistant genes. Third, a plasmid can be modified by the standard recombinant DNA technology. For mammalian expression, a plasmid should have some DNA elements to use endogenous gene expression machinery in mammalian cells. The DNA elements include a promoter, enhancer, intron, therapeutic gene, and a polyadenylation signal (Figure 21.1). For gene expression in mammalian cells, the gene should be transcribed into mRNA and then the mRNA should be translated to a protein. The first step in transcription is binding the RNA polymerase to a plasmid. Binding the RNA polymerase occurs at a promoter that has specific sequences. Also, various transcription factors bind to the promoter at specific sequences and stimulate transcription. The transcription factors interact with other proteins such as RNA polymerase at the promoter and promote formation of pre-initiation complex. An enhancer also stimulates transcription by binding enhancer-binding proteins. The enhancer usually has a stimulation effect regardless of its position. An intron has the effect of stabilizing RNA and enhancing mRNA transport by formation of splicesome in the process of RNA splicing. In addition, most eukaryotic mRNAs have a long chain of AMP called poly(A) at their 3'-ends. This poly(A) is added to mRNA after transcription by poly(A) polymerase. Poly(A) increases the half-life of mRNA and promotes translation by recruiting mRNA to polysomes. A proper combination of these elements affects the expression level of the transgene.

The promoter should be selected depending on the gene therapy purpose and administration route. For systemic delivery, a strong and non-specific promoter is desirable because a high level of gene expression in any tissues will be beneficial for systemic gene therapy. A certain group of therapeutic genes should be localized to maximize the gene therapy efficiency and minimize sideeffects. In this case, a tissue specific promoter should be used for localization of gene expression. In addition, an intron and a polyadenylation signal have an influence on gene expression efficiency in combination with other DNA elements. Many efforts have been made to optimize the DNA elements depending on target diseases and purpose of gene therapy. In this chapter, we will discuss the DNA elements and current progress in this research.

21.2 PROMOTERS

A promoter is located upstream of therapeutic cDNA. The promoter determines the transcription start site and regulates the transcription of a target cDNA. A promoter has various transcription factor binding sites. The transcription factor that binds to a promoter recruits a RNA polymerase complex and induces the initiation of transcription. Some promoters have tissue specific transcription factor binding sites and therefore, the transcription is induced in specific tissues or condition. This characteristic of the promoter enables the tissue specific gene expression for targeting. In this section, we will review conventional and tissue-specific promoters that are widely used for gene therapy.

21.2.1 CONVENTIONAL PROMOTERS

The most widely used conventional promoters are viral promoters such as the simian virus 40 (SV40) promoter, the rous sarcoma virus (RSV) long terminal repeat promoter, and the cytomegalovirus (CMV) promoter. The viral promoters have high transcription rate without tissue specificity. Previously, the activity of these promoters was compared by Guo et al.¹ In the study, the CMV, SV40, RSV, and moloney leukemia virus (MLV) promoters were evaluated for hepatic gene expression in mice using adenoviral vector as a gene delivery vehicle. The results indicated that the CMV promoter/enhancer had the strongest promoter activity.¹ In another study, the SV40, CMV, and RSV promoters were compared in vitro and *in vivo*.² In vitro results suggest that these viral promoters have similar promoter activities. The CMV promoter has a slightly higher promoter activity than the others in HeLa, HepG2, and ECV304 cells. In vivo results were more impressive than in vitro results. In liver and muscle tissue, the CMV promoters.²

The viral promoters have the problem of promoter silencing. After *in vivo* administration, the viral promoters are inactivated rapidly and the promoter activity is decreased significantly within a week. The cause of the promoter silencing has not been fully addressed yet, but it was suggested that it might be

mediated by the CpG motif induced immune response and cytokine overexpression.³ In addition, the reactivation of the silenced viral promoter can be achieved by the inhibitor of histone deacetylase suggesting histone deacetylation is the major cause of the promoter silencing.⁴ The promoter silencing can also be overcome using a mammalian promoter and therefore, the development of a strong mammalian promoter system is one of main requirements for successful gene therapy.³

The most widely used mammalian promoter is the chicken β -actin promoter. The chicken β -actin promoter has a strong promoter activity and therefore, has been used for various kinds of gene therapy. The β -actin promoter was compared to the viral promoters and the results suggest that the β -actin promoter is highly effective in various cell lines in vitro and in liver and muscle *in vivo*.^{2,5} For the enhancement of the promoter activity, the hybrid promoter with the β -actin promoter and the CMV enhancer was also analyzed. The results showed that the CMV enhancer/ β -actin promoter had a higher transgene expression activity in vitro and *in vivo* than the viral promoters.² The hepatic Factor IX (FIX) gene expression driven by the CMV enhancer/ β -actin promoter system. This result also indicated that this hybrid promoter overcame the promoter silencing. This CMV enhancer/ β -actin promoter system has been widely used for various gene therapy researches, including interleukin (IL)-4,^{6,7} IL-10,^{6,8} and IL-12.^{9,10}

21.2.2 TISSUE-SPECIFIC PROMOTERS

21.2.2.1 Liver-Specific Promoters

The α -fetoprotein (AFP) promoter has been widely used as a liver specific promoter.^{11–21} The AFP promoter was first used for liver specific or hepatoma specific expression of thymidine kinase (TK).²² After delivery, expression of TK was limited to the AFP-positive hepatoma cells. The cells that expressed TK were selectively sensitive to the pro-drug. This liver specific expression by the AFP promoter was applied to the expression of diphtheria toxin A (DTA) for the growth inhibition of the hepatoma cells.¹⁶ In this study the AFP promoter/enhancer was inserted upstream of the DTA cDNA. The results showed that the construct with the AFP promoter/enhancer could inhibit the growth of AFP-positive HuH-7 cells, not AFP-negative MKN45 cells. In addition, the combination of the hypoxia responsive enhancer and the AFP promoter ensured the expression of the target gene in hepatoma cells under hypoxia.¹⁷ However, the AFP enhancer/promoter cannot be inserted into an adeno-associated viral vector because of the size limit. The minimum enhancer region of AFP (0.4 kb) and the minimum albumin promoter were linked for the hepatoma specific TK expression.¹¹ Similarly, the AFP enhancer was linked to the phosphoglycerate kinase (PGK) promoter.¹⁸ The PGK promoter with the AFP enhancer induced TK expression in AFP-producing hepatoma cells and suppressed the expression in nonhepatoma cells. This result suggests that the AFP enhancer could give hepatoma specificity to the housekeeping promoter.

The albumin enhancer/promoter was evaluated as a liver specific promoter.^{23–26} The tissue specificity of the promoter was confirmed in vitro and *in vivo* using the β -galactosidase as a reporter gene.²⁴ The results showed that the albumin enhancer/promoter increased transgene expression in dividing hepatocytes of partially hepatectomized mice, but not in nondividing hepatocytes. Therefore, the albumin enhancer/promoter was applied to hepatoma gene therapy.²⁶ In addition, the albumin enhancer/promoter was used for the expression of human serum albumin²⁵ or human α 1-antitrypsin (hAAT).²³ In both cases, partial hepatectomy was performed to induce gene expression. After injection and hepatectomy, the transgene protein in serum was sustained for at least 4 weeks.

The factor IX promoter was also evaluated for the liver-specific therapeutic gene expression.²⁷ By introduction of a prime regulator of the factor IX promoter to the proximal promoter increases promoter activity at least 20-fold over the proximal promoter alone. This promoter was more effective than the SV40 enhancer/promoter system in HepG2 cells.

21.2.2.2 Tumor-Specific Promoters

In the majority of gastric cancers, carcinoembryonic antigen (CEA) is overexpressed. Therefore, gastric cancer specific gene expression was achieved by using the CEA promoter.^{28–38} In this study, the TK gene was controlled by the CEA promoter and the tissue specific TK expression was confirmed in vitro and in vivo.²⁸ In addition, tumor growth was significantly inhibited using this construct. The improvement of the CEA promoter was made by using a chimeric promoter system that was composed of the CMV enhancer and the CEA promoter.²⁹ In vitro study with the chimeric promoter revealed that the promoter system in CEA-producing cell lines was 200-fold more efficient than in CEA-non-producing cell lines.²⁹ The CEA promoter was also used to drive the bacterial cytosine deaminase (CD) gene.^{31,35} A tissue specific CD expression retroviral vector was constructed by using the CEA promoter region. When the vector was injected intraperioneally, survival rates were significantly prolonged. In addition, bone marrow suppression was not detected after injections of the vector. The vector without the CEA promoter showed profound bone marrow suppression. These results revealed that the CEA promoter significantly improved the safety and efficiency of the vector.

The cyclooxygenase-2 (cox-2) promoter was also used as a tumor specific promoter.^{39–41} It was previously reported that the expression of cox-2 is related to colon cancers.⁴² The expression of cox-2 is also undetectable in most tissues under normal physiological condition.⁴³ This expression profile suggests that the cox-2 promoter is applicable to cancer specific gene expression. The cox-2 promoter was employed for the suicide gene therapy of gastrointestinal cancers.³⁹ The expression of TK by the cox-2 promoter was minimized in liver,

showing little activity of the enzyme. However, the enzyme attained high level of the expression in the subcutaneous tumor.

Another tumor specific promoter for gene therapy is the midkine (MK) promoter. Many types of tumors express high level of MK. The tissue distribution study of the MK expression showed that MK is not expressed in liver. Therefore, the MK promoter is one of the good transcriptional regulators for tumor specific expression of the therapeutic genes. The MK promoter was employed to express thymidine kinase specifically in tumors.^{40,44,45} The MK promoter–thymidine kinase system was applied to pediatric solid tumors^{44,45} and ovarian cancer.⁴⁰ After intraperitoneal administration, the MK promoter showed low expression in the peritoneum and liver, which minimized the adverse affects in the normal tissues.⁴⁰

Human telomerase reverse transcriptase (hTERT) is a DNA polymerase for the replication of chromosomal ends. It was reported that telomerase is specifically active in cancer cells, but not in normal cells. hTERT is a key determinant of telomerase activity.^{46–49} Since hTERT is highly active in cancer cells, the promoter was suggested as a transcriptional regulator for tumor specific gene therapy.^{50,51} For the induction of tumor specific apoptosis, the Bax gene was delivered using the hTERT promoter as a transcriptional regulator. As a result, the Bax gene expression was induced specifically in a tumor and suppressed the tumor growth. In addition, the toxicity of the Bax gene expression is minimized in normal cells. The hTERT promoter was also used to drive the caspase-6 gene for cancer specific apoptosis therapy⁵² and thymidine kinase for tumor suicide gene therapy.⁵³

The ovarian specific promoter 1 (OSP1) was evaluated to limit the TK expression in tumor cells.⁵³ The results showed that the stable transfection of the OSP1-TK plasmid to OVCAR3 cells made the cells more sensitive to GCV treatment compared to the parental OVCAR3. In vivo results also showed a longer survival rate in the OVCAR3 survival model. The secretory leukoprotease inhibitor (SLPI) promoter also has ovarian cancer specific gene expression ability.^{54,55} In a study, in which adenoviral vector constructs containing the promoter were used, the SLPI promoter achieved a high level therapeutic gene expression in ovarian cancer cells compared to normal cells. The therapeutic efficacy of the promoter was comparable to the ubiquitous promoters in ovarian cancer cells.

For prostate specific gene therapy, the prostate-specific antigen (PSA) promoter/enhancer has been used.^{56–65} PSA is expressed specifically in hyperplastic and malignant prostatic epithelium. The promoter analysis showed that the PSA promoter is sufficient for transgene expression in prostate tissue.⁶⁶ However, another report showed that the PSA enhancer is required for the tissue specific expression.⁶⁷ The PSA promoter/enhancer system has been used for the suicide gene therapy for prostate cancer.^{57,58,62} An artificial chimeric enhancer (PSES) was developed using two modified regulatory elements of PSA and prostate specific membrane antigen (PSMA).⁶¹ The PSES promoter is highly active in prostate cancer cells, not in nonprostate cancer cells.

Other promoters that were used for tumor specific gene therapy include; the L-plastin promoter, the thyroglobulin (TG) promoter, and the DF3 promoter. L-Plastin is present in >90% of epithelial neoplastic cells and not in normal cells. Therefore, the L-plastin promoter was used to express the cytosine diaminase gene in ovarian or bladder cancer.^{68–70} The TG promoter is a thyroid specific promoter and has been used for thyroid carcinoma gene therapy.^{71,72} The DF3 promoter was used to express the Bax gene specifically in cancer cells.^{73,74}

21.2.2.3 Smooth Muscle Cell-Specific Promoters

For vascular smooth muscle targeting gene therapy, various smooth muscle cell (VSMC) specific promoters have been tested in terms of specificity and strength. One of the most widely used promoters is the smooth muscle α -actin promoter/enhancer. The α -actin enhancer was inserted upstream of the minimal SV40 early promoter, producing a hybrid promoter. The results showed that the hybrid promoter expressed the target gene in the modulation of VSMC proliferation associated with intimal hyperplasia/restenosis.⁷⁵ In addition, the smooth muscle α -actin promoter itself was evaluated for smooth muscle specific gene expression.⁷⁶ In this study, a novel transcriptional repressor, E2F/p56 (a fusion of the C-terminal fragment of Rb (p56) and DP1-binding domains of E2F) was expressed by using the α -actin promoter. The result showed that the gene expression was localized in smooth muscle cells and showed no expression in liver, bladder, or skeletal muscle.

The smooth muscle gamma actin (SMGA) promoter was also evaluated as a smooth muscle cell specific promoter.⁷⁷ An in vitro transfection assay showed that the SMGA promoter limited the gene expression to smooth muscle cells.⁷⁷ Interestingly, the specificity of the SMGA promoter may be due to cell-specific nuclear import of a plasmid. When injected into the cytoplasm, plasmids containing the SMGA promoter were localized to the nucleus of smooth muscle cells, but remained in the cytoplasm in other cells. This nuclear localization of the plasmid may be mediated by the smooth muscle specific transcription factor SRF.

A chimeric smooth muscle specific promoter was produced by combining the smooth muscle myosin heavy chain (MMC) enhancer and the SM22 α promoter.⁷⁸ In vivo experiments with this chimeric plasmid showed that reporter gene expression was restricted to arterial smooth muscle cells with no detectable expression in other cells. The gene expression efficiency of the chimeric promoter was comparable to that of the CMV enhancer/promoter in smooth muscle cells. This result suggests that this strategy is effective to obtain a strong and artificial SMC specific promoter.

21.2.2.4 Skeletal Muscle/Heart Muscle-Specific Promoters

It was previously proven that the direct injection of plasmid DNA to skeletal muscle or heart muscle (myocardium) could elicit high level expression of the therapeutic gene. For more efficient and specific expression in skeletal muscle or heart muscle, various kinds of muscle specific or myocardium specific promoters have been evaluated. As cardiac-specific promoters, the ventricle specific myosin light chain-2 (mlc-2v) and the arterial- and ventricular-specific α -myosin heavy chain (α -mhc) promoters were evaluated.^{79,80} The injection of recombinant adenovirus containing the promoter to cardiac cavity of neonatal rats showed that the high level expression of the reporter gene was achieved in the heart muscle. The gene expression was mainly localized in the heart with low level expressions in the lungs and liver. Direct injection of the vectors to other types of muscle showed only background luciferase activities, confirming heart muscle targeting expression of the gene.

For skeletal muscle specific gene expression, the muscle creatine kinase promoter/enhancer,^{81,82} the human skeletal actin promoter in combination with the CMV enhancer/promoter,⁸³ and the skeletal actin promoter in combination with the creatine kinase enhancer have been evaluated.⁸⁴ The muscle creatine kinase promoter/enhancer was used to drive the expression of the dystrophin gene. The local injection into muscles showed high transgene expression in muscle, but extremely low expression in nonmuscle tissues.⁸¹ After the injection, the human skeletal actin promoter was used to drive the expression of human FIX gene to muscle.⁸³ The skeletal actin promoter itself showed very low expression in vitro and *in vivo*. However, the combination with the CMV enhancer/promoter improved gene expression dramatically, retaining the tissue specificity. Similarly, the human skeletal promoter improved the gene expression in combination with various enhancer.⁸⁴ Therefore, the actin promoter in combination with various enhancer.⁸⁴

21.2.2.5 Pancreas-Specific Promoter

Recently, two pancreas-specific promoters were evaluated in vitro. The pancreatic amylase promoter was tested in vitro.⁸⁵ The amylase promoter can be induced by dexamethasone and insulin. In vitro results showed that the promoter and reporter gene construct retained the inducibility by dexamethasone and insulin. Another example is the insulin promoter.^{86–88} The insulin promoter localizes gene expression in the pancreas. The promoter is a response to glucose concentration. Therefore, the level of transgene expression from the plasmid containing the insulin promoter was dependent on glucose concentration. For the IL-4 expression in the pancreas, the insulin promoter driven IL-4 plasmid was evaluated in vitro.⁸⁷ The results showed that the IL-4 expression is specific in the MIN6 (mouse insulinoma) cells, but not in other types of cells.

21.2.2.6 Lung-Specific Promoters

The lung specific promoters have been used for gene therapy for many genetic and acquired diseases of the lungs. The first example of a lung specific promoter is the human surfactant protein B (SP-B) promoter.⁸⁹ A reporter plasmid was constructed using the human SP-B promoter. While the gene expression by the Rous sarcoma virus promoter was detected in various organs such as the lungs, trachea, heart, liver, and esophagus, the SP-B promoter was localized in the lungs, specifically in air space-lining cells. The SP-B promoter was also used for lung cancer treatments.⁹⁰ Tumor bearing mice were produced by injection of H441 cells, a human cancer cell line in which the SP-B promoter was active, or Hep3B liver cancer cells. After injection of the vector containing the SP-B promoter, tumor suppression was observed in the mice with H441 tumor. The vector had minimal effect on Hep3B tumor confirming tissue specificity of the SP-B promoter.

21.2.2.7 Endothelial Cell-Specific Promoters

Tumor growth is usually dependent on blood supply and development of tumor vasculature. Therefore, the targeting angiogenic tumor vasculature is one approach for tumor targeting. For this requirement, endothelial cell specific promoters were evaluated. The human prepro-endothelin-1 (PPE-1) promoter was tested with a reporter gene.⁹¹ An in vitro assay showed that the promoter expressed the transgene mainly in primary breast microvascular endothelial cells. Similarly, the murine PPE-1 promoter showed the same targeting gene expression ability.⁹² An in vitro study with the murine PPE-1 promoter showed high expression in bovine aortic endothelial cells. In an *in vivo* study, the gene expression was localized in the aorta and vascularized tissues such as heart, kidney, lung and pancreas tissues after systemic administration. In addition, in mice bearing Lewis lung carcinoma, the highest transgene expression was detected in angiogenic endothelial cells of the metastasis.

The plasminogen activator inhibitor 1 promoter and *fms*-like tyrosine kinas-1 (FLT-1) promoter were evaluated for vascular endothelial cell targeting.^{93,94} PAI-1 is the inhibitor of tissue-type plasminogen activator and urokinase-type plasminogen activator. PAI-1 is secreted from vascular endothelial cells. The chimeric promoter composed of the PAI-1 promoter and the CMV enhancer showed the transgene expression in vascular endothelial cells at a comparable level to the chicken β -actin promoter/CMV enhancer system.⁹³

The FLT-1 promoter also showed high specificity in the transgene expression in endothelial cells.⁹⁴ The systemic administration or ex vivo study of the vector containing FLT-1 promoter showed endothelial cell specific transgene expression in an intact vein and extremely low-level gene expression in the liver.

21.2.2.8 Brain/Neuron-Specific Promoter

A wide range of brain/neuron specific promoters have been evaluated. One of the most widely used promoters is the synapsin-1 (SYN1) promoter.^{95–98}

Glover et al. constructed an expression cassette containing the human SYN1 promoter and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE).⁹⁵ An *in vivo* study revealed that the 3' addition of the WPRE to the SYN1 promoter greatly increased the transgene expression without loss of tissue specificity. The SYN1 promoter was used to drive the expression of Bcl-XL gene for the treatment of the brain infarction following focal cerebral ischemia.⁹⁶ The SYN1 and Bcl-XL constructs were injected to striatum. After ischemic induction, the injury by hypoxia was greatly reduced when the Bcl-XL gene was expressed by the SYN1 promoter. The long-term transgene expression was also achieved with tissue specificity in the rat brain by using the SYN1 promoter.⁹⁷

The rat preproenkephalin (PPE) promoter was used for the gene expression in the brain.⁹⁹ PPE is produced in the brain in a highly specific pattern. The local administration of the vector revealed that the 2.7 kb fragment of the PPE promoter was enough to localize the transgene expression in several brain regions, where endogenous PPE was expressed.

The neuronal specific enolase (NSE) promoter was used to control the Fas ligand (FasL) expression for the treatment of a brain tumor.¹⁰⁰ The NSE promoter limited the transgene expression in neurons and glial cells. The NSE promoter was compared to various promoters in terms of strength.¹⁰¹ The results showed that the 1.8 kb NSE promoter had a higher promoter activity in the striatum and the hippocampus than other promoters such as the CMV promoter. The activity of the 1.8 kb NSE promoter was further enhanced with WPRE. With WPRE, the gene expression by the NSE promoter was prolonged, compared to the NSE promoter without WPRE.

Other brain specific promoters include the human glial fibrillary acidic protein (GFAP) promoter¹⁰² and the latency associated transcripts (LAT) promoter.^{103,104}

21.2.2.9 Other Tissue-Specific Promoters

The human cytokeratin 18 (KRT18) promoter was used for cystic fibrosis gene therapy.¹⁰⁵ The gene delivery by a cationic liposome of the plasmid showed the localized gene expression in nasal and bronchial epithelium, airway submucosal glands, gall bladder, and kidneys. The expression was low in other organs such as the gut and no expression was detected in the liver and alveolar lung.

Hematopoietic stem cell specific gene therapy employed the integrin aIIb promoter,¹⁰⁶ the human ankyrin (Ank) promoter,^{107–109} and the GATA-1 enhancer.^{108,110} The mouse phosphoglycerate kinase I (PGK), elongation factor-1 (EFS), and rhodopsin (Rho) promoters were evaluated for retinal cell gene expression.¹¹¹ The results suggest that the PGK promoter localizes the gene expression in the retinal pigment epithelium while the EFS promoter is expressed broadly in the retina. In addition, the Rho promoter expressed the transgene in photoreceptors. The CD4 mini-promoter/enhancer was used to express the gene in peripheral blood lymphocytes.¹¹² The myo7a promoter

localized gene expression in the hair cells of the inner ear.¹¹³ The human calponin promoter expressed the transgene in human soft tissue and bone tumors.¹¹⁴

21.2.3 INDUCIBLE PROMOTERS

One of the most widely used inducible promoters is the hypoxia inducible promoter. The most solid tumor has ischemic tissue and the ischemic diseases such as ischemic heart disease, hindlimb ischemia, and stroke have severe hypoxic regions. Therefore, a hypoxia inducible promoter is a requirement for the specific and safe treatment of ischemic diseases. A hypoxia inducible promoter usually includes hypoxia responsive elements (HRE) in combination with a basal promoter. Virtually all hypoxia inducible promoters are regulated by hypoxia inducible factor-1 (HIF-1), which is a key regulator of hypoxia response in cells. HIF-1 is stabilized and increases the target gene expression under hypoxia. Therefore, hypoxia responsive promoters usually have many copies of HIF-1 binding sites. The hypoxia inducible plasmid was constructed with HRE in combination with a basal promoter such as the SV40 minimal or CMV minimal promoter.^{115–118} The HRE was optimized and referred to Oxford Biomedica hypoxia response element (OBHRE).¹¹⁹⁻¹²¹ The OBHRE was linked to the CMV promoter for hypoxia inducible high gene expression. For hypoxia inducible heart specific expression, the HRE-MLC2v promoter was constructed.¹²²

The VEGF promoter is induced under hypoxia. Therefore, the VEGF promoter was also used for hypoxia inducible gene expression.^{118,123,124} Recently, the RTP801 promoter was suggested as a strong hypoxia inducible promoter.¹²⁵ The RTP801 promoter plasmids were constructed with a reporter gene or the VEGF cDNA. An in vitro study suggested that the RTP801 promoter had stronger gene expression efficiency under hypoxia than erythropoietin (Epo)/SV40 minimal promoter system. Since the viral promoters have promoter silencing effect, the hypoxia inducible system from mammalian promoters may improve the gene expression profile under hypoxia.

For insulin concentration dependent gene expression, the phosphoenolpyruvate carboxykinase (PEPCK) promoter was evaluated.¹²⁶ The activity of the PEPCK promoter is down-regulated by insulin. A plasmid was constructed, in which the insulin gene expression was controlled by the PEPCK promoter. After addition of exogenous insulin to the cell culture medium, the cells expressed insulin at a remarkably decreased level.

For glucose concentration dependent induction and insulin dependent inhibition of the gene expression, the combination of three stimulatory glucose responsive elements from the rat liver pyruvate kinase (L-PK) promoter and an inhibitory insulin response element of the insulin-like growth factor binding protein-1 (IGFBP-1) promoter was produced.¹²⁷ This hybrid promoter retained the responsiveness to blood glucose levels and insulin dependent reduction of the gene expression. This type of the promoter may be useful for

the self-regulatory insulin gene therapy. Similarly, the glucose-6-phosphatase promoter was used for the self-regulatory insulin gene therapy.¹²⁸

Another type of an inducible promoter is a heat inducible promoter. For heat induction of gene expression, the heat shock protein 70 (hsp70) promoter was intensively studied.^{129–131} The hsp70 promoter is induced by about 500–1000 fold over background by moderate hyperthermia (39 to 43°C). In previous trials, the heat-inducible interleukin 12 or tumor necrosis factor α gene was delivered and then heated (42°C) for 30 min.¹³² The results showed that the gene expression was induced 13,600 or 6.8×10^5 fold over the background respectively. Lipinski et al. constructed a vector including two expression cassettes; the *E. coli* nitroreductase gene driven by the hsp70 promoter and a p53-inducible lac repressor gene.¹³⁰ Therefore, nitroreductase inhibited tumor growth in tumor cells not containing wild-type p53. In the cells retaining wild-type p53, the expression of nitroreductase was significantly reduced. The human multidrug resistance gene (mdr1) promoter was also employed for heat inducible gene expression.¹³³ After heat at 41.5°C or 43°C

A tetracycline regulatory system was widely investigated for the application to gene therapy.¹³⁴ The mechanism of the tetracycline on–off system was presented in Figure 21.2. In the tet-on system, tetracycline binds to a tetracycline binding domain and induces the conformational change of the TetR-VP16 fusion protein. The TetR binds to a tetracycline response element in the promoter and the VP16 transactivation domain drives the transcription



FIGURE 21.2 The tetracycline on-off system. In the tet-on system, tetracycline binds to a tetracycline binding domain and induces the conformational change of the TetR-VP16 fusion protein. The TetR binds to a tetracycline response element in the promoter and the VP16 transactivation domain drives the transcription of the target gene.

of the target gene. In the tet-off system, the tetracycline binding to TetR domain inhibits its binding to TRE, resulting in transcriptional inhibition.

Other inducible promoters include the early growth response-1 (Egr1) promoter as a cisplatin inducible promoter,¹³⁵ the glucocorticoidsensitive promoter,¹³⁶ and the human serum amyloid A2 promoter as an inducible promoter during inflammation.¹³⁷

21.3 INTRONS

Most natural genes contain introns as well as exons, which are spliced out in the process of the mRNA maturation. Previous reports showed that the intron sequence in a pre-mRNA increased the yield of gene product in several eukarvotic systems.^{138–140} was suggested that pre-mRNA forms It the splicesome assembly in the process of post-transcriptional splicing. The splicesome assembly stabilizes the mRNA and facilitates migration of the mRNA to the nuclear pore. The effects of the intron elements have not been studied intensively. Some reports show that intron A of human FIX gene might increase the gene expression of the hFIX or FVIII gene.¹⁴¹⁻¹⁴³ The systemic analysis of the intron element in terms of gene expression efficiency was performed by Xu et al.² In this study, the insertion of intron A increased the gene expression level by 2- to 50-fold in plasmid vectors.^{2,144} A chimeric intron was produced using the 5'-donor splice site from human β -globin intron 1 plus the 3'-acceptor splice site from the intron of an immunoglobin gene heavy chain variable region. This chimeric intron was compared to intron A from the human cytomegalovirus immediate-early 1 gene.² Insertion of intron A into a plasmid increased the gene expression level remarkably compared to that of the chimeric intron. However, the effect of the intron in vivo administration was not as efficient as in vitro study. In muscle, the increase of intron A was about twice as much compared to the gene expression without any intron. This study showed that the intron could increase the gene expression, but the intron should be carefully designed to optimize the gene expression.

21.4 POLYADENYLATION SIGNAL

A polyadenylation signal is required for the proper termination of transcription and stability of mRNA. In addition to these effects, poly(A) sequence can affect overall gene expression efficiency. The analysis of poly(A) sequences was performed by Xu et al.² In this study, the SV40, bovine growth hormone (BGH), and mRBG poly(A) sequences were compared to each other. In vitro studies showed that the SV40 poly(A) was the most effective in Hela or EVC304 cells in combination with the chicken β -actin promoter. However, the mRBG poly(A) with the CMV promoter and intron A was the most effective in terms of gene expressions. In vivo administration showed that the BGH poly(A) is the most effective in the liver, and that the SV40 poly(A) was the most effective in muscle. The poly(A) sequence should be carefully chosen depending on the administration route and target tissue.

21.5 MULTIPLE GENE EXPRESSION

In many cases, expression of more than one gene is required to effectively treat a disease. Therefore, the multigene expression system has been studied to optimize gene expression efficacy. The most useful system for multigene expression is internal ribosome entry site (IRES). IRES enables capindependent translation of mRNA. In the IRES system, two cording regions are transcribed from a single promoter and both genes are translated from a single mRNA. This IRES system is more useful in viral vector than two promoter systems, since two identical promoters may induce homologous recombination. IRES from the encephalomyocarditis virus (ECMV) was employed to encode two genes in a plasmid.^{145–151} One example of IRES application is the interleukin-12 (IL-12) expression plasmid, pIRES-mIL12. IL-12 has two subunits, p35 and p40 and the two subunits should be co-expressed for efficient IL-12 expression. For this purpose, a IL-12 plasmid with IRES was evaluated.^{10,152} An in vivo administration of pIRES-IL12 expressed the two subunits effectively in a tumor and suppressed the tumor growth.^{9,10} The second gene expression mediated by IRES is not as efficient as the first gene. Therefore, the improved IRES system is required for maximum efficiency. Recently, it was suggested that IRES from human genes has better translation ability than IRES from ECMV.¹⁵³ In addition, synthetic oligonucleotides were screened to select efficient IRES, which improved the efficiency of the IRES.154

The simple multigene expression system is two expression cassettes in a plasmid. In the example of IL-12, p35 and p40 should be expressed equally. Therefore, p2CMV-IL12 was used to express p35 and p40 using two independent expression cassettes.¹⁵⁵ In this plasmid, the expression of p35 and p40 were driven by two separate CMV promoters/enhancers. An in vitro study showed that these two promoter systems had higher IL-12 expression than the IRES system. Similarly, for the prevention of type 1 diabetes, the IL-4 and IL-10 co-expression plasmid was developed using two independent CMV promoters/enhancers.¹⁵⁶ In vitro and *in vivo* studies revealed that this co-expression plasmid with two promoters worked better than a two plasmids system. However, if the two expression cassettes are located too close to each other, the RNA polymerase from the first cassette may interfere with binding of transcription factors to the downstream promoter. The two promoter plasmid should be carefully designed to avoid this negative effect of the position of the promoters.

21.6 TWO STEP TRANSCRIPTION AMPLIFICATION (TSTA) SYSTEM

Tissue specific promoters often suffer from the low transcription activity, compared to conventional promoters. In addition, a nonviral gene delivery system requires high level promoter activity for gene therapy efficacy. Therefore, the transcription amplification systems have been developed to



FIGURE 21.3 Two step transcription amplification system. In the two step transcription amplification system, the first plasmid contains a conventional or tissue-specific promoter to express Gal4-p65 fusion protein. The expressed Gal4-p65 fusion protein then binds to Gal4 binding motif of the target plasmid and activates the transcription.

increase the gene expression level. The transcription amplification is achieved by two step transcription (TSTA, Figure 21.3). The most widely studied TSTA is the Gal4-p65 system.^{33,157–159} The first plasmid contains a conventional or tissue-specific promoter to express Gal4-p65 fusion protein. The Gal4-p65 fusion protein then binds to Gal4 binding motif of the target plasmid and activates the transcription. This transcription in TSTA is amplified by 40–2000 times compared to the simple SV40 promoter system.^{33,159–161} This system was applied to; the hypoxia inducible system,¹⁶⁰ the tumor specific gene expression system³³ and the androgen responsive system.¹⁵⁹

Another transcriptional amplification system is composed of T7 promoter and T7 RNA polymerase. The first plasmid expresses the T7 RNA polymerase. The expressed T7 polymerase binds to the T7 promoter on another plasmid and increases the transcription rate.^{162,163} The gene expression remarkably increased with this system. However, one of the main problems of this system is cytotoxicity of T7 RNA polymerase. The T7 RNA polymerase is not an endogenous protein in mammalian cells and therefore, may induce immunogenecity and cytotoxicity.

21.7 NUCLEAR TARGETING

Nuclear targeting is an important consideration for nonviral gene delivery because nonviral gene delivery carriers usually do not have delivery machinery to the nucleus unlike viral carriers. Many studies were performed to conjugate the nuclear localization signal peptide to carriers. Another approach has been made using DNA element which is known as a nucleus targeting sequence. It was previously reported that the SV40 enhancer has the sequence for the nuclear import. The study showed that SV40 DNA was efficiently transported into the nucleus while other bacterial plasmids were not.¹⁶⁴ The study suggested that the 72-bp repeats of the SV40 enhancer facilitate maximal transport. This result was confirmed by the study of Li et al.¹⁶⁵ The insert of the SV40 enhancer at the 3' end of poly(A) signal increased the gene expression as much as 20-fold, compared to the plasmid without the SV40 enhancer. This result was confirmed in an *in vivo* study.¹⁶⁶ The *in vivo* gene expression by the plasmid with the SV40 enhancer increased 10-fold at 2 days and 40-fold at 3 days, compared to a plasmid without the SV40 promoter was transported into the nucleus.

Another nuclear targeting method was developed using nuclear factor (NF)- κ B binding site.¹⁶⁷ NF- κ B is a transcription factor that is localized in cytoplasm as an inactive protein complex with I κ B. In response to exogenous stimulation, NF- κ B was dissociated from I κ B and transported to nucleus. To use this transport machinery as a nuclear targeting method of a plasmid, multiple copies of the NF- κ B binding sites were incorporated into a plasmid. Without a stimulator, the incorporation of the NF- κ B binding sites increased nuclear transport from 2.6- to 5.8-fold. This enhancement in basal activity may be due to low-level migration of NF- κ B into the nucleus. When a stimulator was used, the transport was further enhanced and the transfection activity reached up to 35 times better results compared to the plasmid without the NF- κ B binding sites. This result suggests that a transcription factor mediated nuclear-transport is a useful way to increase transfection efficiency

21.8 CpG DEPLETED PLASMID AND MINICIRCLE PLASMID

It is generally accepted that bacterial DNA causes inflammation. This immunotoxicity is due to the unmethylaed CpG motifs in a particular base context.^{168,169} CpG content and methylation distinguish vertebrate and bacterial DNAs.¹⁷⁰ In vertebrate genomes, CpG dinucleotides are suppressed. The number of CpG motifs in mammalian genome is about one quarter of that of bacterial DNA. In addition, the CpG motifs in vertebrate genomes are highly methylated, while the motifs are not methylated in bacteria. Therefore, the immune system recognizes this difference and activates an immune response. These CpG motifs in bacterial DNA lower the efficiency of the transgene expression. Methylation of bacterial DNA decreased the level of inflammatory cytokines and increased the transgene expression.¹⁷¹ One of the approaches to reduce this negative effect of bacterial DNA is to eliminate the sequence. Yew et al. constructed a CpG-depleted plasmid by replacing large portions of the plasmid with a synthetic, non-CpG sequence.¹⁷² The systemic administration of the CpG-depleted plasmid reduced the levels of inflammatory cytokines, and decreased liver damage. In addition, the transgene
expression persisted longer than the original plasmid. In other reports, Minicircle DNA was constructed and evaluated for reduction of CpG motifs in the bacterial DNA part in a plasmid.^{173–175} Since bacterial DNA has about four times more CpG motifs than mammalian DNA, the elimination of the bacterial DNA from a plasmid can remarkably reduce this negative effect and increase transgene expression. Using restriction enzyme method to produce minicircle DNA is very tedious. A more efficient way to produce minicircle DNA was developed using phage λ integrase or cre recombinase. The minicircle DNA was obtained in E. coli by att site-specific recombination mediated by the phage λ integrase. The phage λ integrase excised the expression cassette from the bacterial plasmid sequences and produced the minicircle DNA. The purified minicircle DNA was evaluated in terms of gene expression and compared to the original plasmid containing the bacterial DNA.^{173,174} An in vitro experiment showed that the gene expression was increased by 2- to 10-fold, compared to the original plasmid. In vivo experiments showed more remarkable results. The gene expression in muscle and tumors resulted in 13 to 50 times higher gene expression with minicircle DNA than the original plasmid. Another approach to produce minicircle DNA is to use cre recombinase. The cre-mediated excision of the entire bacterial vector sequence significantly increased over the original plasmid in gene expression.175

21.9 CONCLUDING REMARKS

A plasmid has advantages as a carrier of genetic information. Therefore, it has been widely used for nonviral gene therapy. Recently, the DNA elements in plasmid have been optimized to increase therapeutic efficacy and safety using various promoters, introns, and polyadenylation signals. Systemic analysis of the DNA elements has not been performed and available information of the elements is limited. For example, the tissue-specific promoters have different specificity, strength, and persistence. In addition, combination of different promoters, introns, and polyadenylation signals changes the specificity and strength of the gene expression system. Therefore, plasmid should be carefully designed with various DNA elements for a certain disease. This suggests that the systematic analysis of the DNA elements should be carried out to construct a tailor-made therapeutic plasmid. Therapeutic genetics that study therapeutic plasmids is now an important field in gene therapy. Therapeutic plasmids should be developed along with the development of gene carriers. In addition, the development of molecular biology will eventually make it possible to construct a tailor-made therapeutic plasmid to each disease.

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22 Design Elements of Polymeric Gene Carriers

Joon Sig Choi and Jong-Sang Park

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22.1 INTRODUCTION

Plasmid-based gene therapy is a promising protocol for treating certain human diseases for which other clinical trials are ineffective or unavailable.¹ During the past decade, intensive research and development in multidisciplinary fields, such as chemistry, molecular biology, pharmaceutics, biochemistry, chemical engineering, and medicine, have been directed towards devising optimized and more effective methods for transferring therapeutic genes into cells and for eventual use in human clinical settings. Several successful clinical trials reported so far involve viral vector systems (retro-viruses, adenoviruses) that provide efficient transduction and high levels of gene expression. However, their clinical safety and effectiveness are still hampered by their major drawbacks such as inherent toxicities, short- and long-term risks such as generation of host immune responses, and the possibility of inserted genes combining with activation of oncogenes.² For these reasons, nonviral vector systems that are considered as alternative tools to such risky viral vectors have been introduced and tested for their potential to be safer, and more desirable methods for gene delivery and clinical gene therapy.³ The nonviral vector systems are generally composed of either naked plasmid DNA or various kinds of DNA-complexing agents such as cationic liposomes and polycationic polymers. However, currently available synthetic nonviral vector systems have been beset with many problems, such as inefficiency, cytotoxicity, and water-solubility problems that limit the many possible applications for their *in vivo* use. Consequently, few nonviral vectors have been so far successful in clinical trials.

The focus of this chapter is to examine briefly the key elements that are generally required in the field of nonviral gene delivery research rather than to scrutinize all the polymer-based gene delivery vectors. We wish to provide the basic and newest criteria that should be addressed before designing novel polymers en route to creating powerful, safe, and reliable nonviral vector systems.

22.2 TRADITIONAL POLYCATIONIC CHARGED-BASED POLYMERS

22.2.1 STRUCTURE OF CATIONIC POLYMERS

Various types of cationic polymers, i.e., linear, dendritic, cross-linked, branched, and network-type polymers, have been introduced and tested for their potential applicability to the field of gene therapy. The structures of the representative polycationic polymers are shown in Figure 22.1. Some linear cationic polymers were found to be promising at the first stage, but unexpected characteristics such as water-solubility of DNA complexes, low level of transfection efficiency, and inherent cytotoxicity, limited their use as *in vivo* gene carriers.⁴ However, polycationic dendrimers are still very attractive, because of their well-defined structure and ease of controlling their surface functionality for the design of biomedical applications.^{5,6} Already, both polyamidoamine (PAMAM) and polyethylenimine (PEI) dendrimer were tested for their potential utility and these have exhibited relatively high transfection efficiency in vitro and in vivo.^{5,7,8} One of the important features of PEI and PAMAM dendrimers is that they are composed of tertiary amine-containing backbones, which possess pH-sensitive functionality. The so-called "proton sponge effect" or "endosome buffering hypothesis" is the mechanism generally advanced to account for the high transfection efficiency of the polymers.⁷ In addition to the endosome buffering functionality, another merit of designing polymers of globular structure rather than linear or branched or flexible structures is that globular polymers show reduced cytotoxicity.9



FIGURE 22.1 Structures of the traditional polycationic polymers. (a) poly-L-lysine. (b) linear polyethylenimine. (c) Branched polyethylenimine. (d) Polyamidoamine dendrimer (PAMAM) generation 4.

22.2.2 Types of Chargeable Moiety

One of the basic requirements for charge-based complex formation with polyanionic DNA is that the polymer should contain polycationic charge properties. Usually, the types of chargeable moiety are primary, secondary, tertiary, and quaternary amine derivatives. The tertiary amine-containing polymers are less effective at condensation of plasmid DNA than primary or quaternary amine-containing polymers because of the lower degree of protonation at physiological conditions. Interestingly, quaternary amines could bind with DNA effectively, even more strongly than the primary amines–DNA interaction. However, it is noteworthy that a very poor transfection efficiency was observed for the quaternized polycationic polymers.¹⁰

The condensation of plasmid DNA into nano-sized particles contributes to both physicochemical properties and stability against enzyme action. The formation of polycationic particles with DNA increased transfection efficiency in vitro because they could bind to the negatively charged cell membranes and, sometimes, they also could physically come into contact with the cell surface through sedimentation. However, for *in vivo* transfection trials, the systems are inefficient because of the net positive charges of the complexes and formation of large particulates that also reduce the mobility of complexes significantly. The charged particles interact with proteoglycans that are composed of a core protein and sulfated or carboxylic glycosaminoglycans (GAGs) conjugated to the protein. So the transfectivity of the positively charged DNA complexes may be affected by the extracellular polyanionic GAGs that can interact with the complexes, thereby inhibiting their mobility in tissue, and their targeting to some specific cells in vivo.

Recently, our group has reported another PAMAM dendrimer-based gene delivery system.¹¹ PAMAM-OH dendrimer is structurally identical to PAMAM dendrimer except that all terminal functional groups are hydroxyl groups not primary amines. So PAMAM-OH could not form charge-based polyplexes with DNA by itself and shows a deficiency in transfection. The internal quaternary amines were generated by methylation and turned the polymer into a transfection-competent vector preserving the zeta potential of the DNA complexes neutral (Figure 22.2).

22.2.3 EFFECT OF CHARGE DENSITY AND MOLECULAR WEIGHT

The physicochemical properties of the representative polycationic polymers generally used in gene delivery experiments are presented in Table 22.1. Poly(L-lysine) (PLL), which was used as a standard basic polymer in the early stages of polycationic polymer-mediated gene delivery experiments, possesses polypeptide backbones and only primary amines at the terminal ends of the side groups of each lysine unit. Due to its deficiency of endosome buffering moiety, the polymer needs an additional chemical, chloroquine, to achieve gene expression. With respect to the charge density (one cationic charge per molecular weight of monomer unit), PLL and PAMAM are smaller in charge density compared to PEI (0.0097 for PLL; 0.0087 for PAMAM, vs. 0.0238 for PEI). As shown in Table 22.1, PEI has higher transfection efficiency than other transfection reagents due to its endosome buffering and nucleus targeting ability and it shows higher toxicity to cells due to its high charge density and nondegradability. Therefore, cytotoxicity and transfection efficiency are



FIGURE 22.2 (a) Synthesis of quaternized PAMAM G4 dendrimer. (b) Zeta potential values of PAMAM G4/DNA complex (\Box). and 0.97 QPAMAM-OH/DNA complex (\blacklozenge). (c) Reporter gene expression assay at charge ratio 6 (+/–).

believed to be a function of molecular weight, charge density, degradability, and polymer structure.

22.2.4 DEGRADABILITY

In view of the cytotoxicity and DNA release in response to specific environmental stimuli, i.e., hydrolysis, enzymatic digestion, pH difference, and reduction potential difference, degradation functionality of the polymer is an essential feature of efficient polymeric gene carriers. The degradable

TABLE 22.1

Physicochemical properties of representative polycationic polymers generally used in gene delivery experiments

Polymer	Molecular weight (kDa)	Order of amines (degree)	Cationic charge/Monomer (+/Da) ^a	Structure	Degradability	In vitro toxicity ^b	In vitro transfection efficiency ^b
Poly-L-lysine	19.2–36.6	1	0.0097	Linear	Poor, polypeptide	+++	$+^{c}$
PAMAM dendrimer G4	14	1, 3	0.0087	Globular, dendrimer	Poor, polypeptide	+	++
Linear PEI	22	1, 2	0.0238	Linear	Not degradable	++	+++
Branched PEI	25	1, 2, 3	0.0238	Branched, dendrimer	Not degradable	+++	+++

^aDetermined as one cationic charge per molecular weight of monomer unit.

^bArbitrary units, compared with branched PEI polymer.

^cIn the presence of chloroquine.



FIGURE 22.3 Scheme of polymer/DNA complex formation and DNA unpacking from the degradable complexes.

chemical linkages include esters, carbamates, disulfides, ortho esters, acetals, glycosides, and related functional groups. As demonstrated in Figure 22.3, the strategy is to construct degradable polyplexes such that the functional particles may attain a lower level of polymer-mediated toxicity and a higher level of gene expression through increased DNA release from the complexes after reaching target sites compared to nondegradable or poorly degradable polymer-based systems.

We and others have designed various degradable polycationic polyester polymers, which can self-assemble with plasmid DNA forming nanoparticles and show gene transfer potency in vitro.^{12–16} The two major synthesis methods reported are: (i) melting condensation of diols and carboxlic acid derivatives, and (ii) polycondensation using Michael addition involving diacrylates and amine groups. The brief polymerization schemes are represented in Figure 22.4.

Akinc et al. and Lynn et al. reported the first approach to developing a library of parallel synthesis and screening methods, and suggested effective transfection efficiency by poly(β -amino esters).^{17,18} The library was constructed using 140 structurally diverse polymers. Among them, only half of the members, i.e., 70, were soluble in water, which made it possible to characterize them further for DNA condensation and transfection. In addition, 56 polymers of 70 could form complexes with DNA, whereas 14 members could not.

In addition to the relatively low-throughput synthesis and characterization methods, a high-throughput manipulation method was successfully introduced for preparing a library of 2350 structurally diverse, degradable cationic polymers with the aid of liquid handling automation.¹⁹ This provided large amounts of structure-function information.

Another degradable system is composed of disulfide linkages that are sensitive to the reduction potential difference between inside and outside of the cell membranes. The basic idea is that as the concentration of the reduced form of glutathione is 500 times higher than that of the oxidized form in red cells,²⁰ the polymers composed of disulfide bonds could be degradable



FIGURE 22.4 Synthesis of cationic polyester polymers by (a) melting condensation of diols and carboxlic acid derivatives, and by (b) polycondensation using Michael addition of diacrylates and amine-containing monomers.

due to reduction of the linkages releasing DNA from the complexes. Many groups have studied the redox-triggered DNA releasable polymeric carriers that contain disulfide linkages, and the DNA complexes are susceptible to thiolysis, which influences the multivalent interaction between the cationic polymers and the complexed DNA so that the DNA could migrate into the cytoplasm. Gosselin and colleagues synthesized reversibly cross-linked polyplexes for gene delivery.^{21,22} They used PEI polymer and employed homobifunctional amine reactive cross-linking reagents to introduce disulfide linkage inside the polyplexes. Some groups have also tried to prepare cysteine-containing polypeptides and evaluated them for polyplex formation and transfection efficiency.^{23,24} Despite the disulfide-containing polymers showing enhanced complexation with DNA, as well as increased stability and degradability, the real mechanism is still unclear. One possible explanation could be that the reduced form of glutathione might react with the polyplexes and the DNA released into the cytoplasm is subjected to the intracellular machinery of gene expression. Recently, another hypothesis was also proposed that protein disulfide isomerase (PDI), which was reported by Mandel and colleagues in 1993,²⁵ might play an important role in the thiolysis of biomacromolecules that cannot diffuse through the plasma membranes.²⁶

Wang and his colleagues have reported novel biodegradable cationic polymers containing a phosphate backbone and positively charged moiety.^{27,28} The polymer backbone was prepared by ring-opening polymerization of 4-methyl-2-oxo-2-hydro-1,3,2-dioxaphospholane using triisobutylaluminum

as an initiator. Further, modification of phosphorus atom leads to the possibility of introducing of mono- or multiple-cationic charge functionality for electrostatic interaction with plasmid DNA. Even though the transfection efficiency of the polymers was low and it needs an additional endosome disruptive agent, such as chloroquine, in vitro experiments to obtain elevated levels of gene expression, biocompatibility and much lower cytotoxicity are considered to be the key features of the polymers for future in vivo application.

22.3 HYBRID OR GRAFT POLYMERS FOR MULTIFUNCTIONALITY

22.3.1 FORMATION OF STEALTH COMPLEXES: PEGYLATION

One of the recent strategies attempting to overcome such problems is to link or conjugate polycationic polymers with a hydrophilic polymer, poly(ethylene glycol) (PEG). PEG shows many useful characteristics, such as high solubility in water, non-immunogenicity and improved biocompatibility. PEG has been widely used for delivery of many water-insoluble small molecular weight and proteins drugs. Moreover, PEG is often used as a spacer between targeting ligand and polymeric carriers.²⁹ For the preparation of synthetic gene delivery carriers, PEG has also been coupled to numerous polycationic polymers, such as poly(L-lysine), dendrimers, polyspermine, and polvethylenimine.³⁰⁻³⁸ Therefore, the conjugated PEG helps the reagents to improve their half-life in the bloodstream, to increase solubility, and to reduce the immune reaction of complexes with DNA. In addition, receptor-mediated endocytosis can be realized by introducing specific targeting ligands at the end of PEG. The PEG chain serves as a flexible spacer between ligands and receptors. The formation schemes of PEG-coated polyionic complexes between PEG-conjugated copolymers and DNA are depicted in Figure 22.5.

22.3.2 BIOCOMPATIBILITY ISSUES

Biological evaluation of polymeric gene carriers should be performed to determine the potential risks of toxicity resulting from contact of either the polymer itself or the component materials after degradation with cells and with the body. There are three major considerations proposed by the FDA for medical devices, which are considered to be also applicable to polymeric gene carrier materials (http://www.fda.gov/cdrh/devadvice/pma/ special_considerations.html). First, the polymers or the released constituents after degradation of the polymers should not cause any adverse local or systemic effects. Second, they should not be carcinogenic. Finally, they should not produce adverse reproductive and developmental effects. In addition, the biological and chemical characteristics of polymers and the nature, degree, frequency, and duration of their exposure to the local cells or the body must be considered. Therefore, careful evaluation of any new polymer intended for clinical use should be based upon sufficient data from systematic testing



FIGURE 22.5 Polyplex formation of PEG-conjugated hybrid copolymers with DNA. (a) AB type copolymer: linear–linear copolymer. (b) AB type copolymer: linear–dendrimer copolymer. (c) ABA type: dendrimer–linear–dendrimer copolymer.

to ensure that the polymers are safe for *in vivo* use.³⁹ One of various ways to improve the biocompatibility of polymers is to modify the polymers with hydrophilic polymers, such as PEG, which are reported to reduce the surface adsorption of proteins such as fibrinogen, albumin, or thrombin.⁴⁰

22.3.3 GRAFT OR SURFACE FUNCTIONALITY

To supply additional functionalities on the polymer, which is monofunctional, difunctional, or multifunctional, is one of the important aspects in the development of smart polymeric gene carriers. As Han and colleagues reported,⁴¹ the construction of polyplexes with various functionalities may be accomplished as presented in Figure 22.6.

As an example of a pH-sensitive water-soluble DNA-containing nanoparticle system, Choi et al. reported recently that water-soluble DNA-containing nanoparticles, which are composed of novel pH-sensitive polymeric lipid as well as cationic lipid, showed a much increased level of gene expression



FIGURE 22.6 Types of polymer-mediated complex formation with DNA. (a) Homopolymer type. (b) Graft copolymer type. (c) Functional graft copolymer type. (d) Multifunctional copolymer type.

compared to that of pH-insensitive nanoparticles.⁴² The pH-sensitive polymeric lipid contains an acid-labile linker, ortho ester bond, which is presumed to quickly degrade when the particles are endocytosed by cells and are subjected to low-pH conditions of endosomes. Systematic investigations of the chemical modifications of PEI, the most effective polymeric gene carrier commercially available up until now, and the resulting effects on the characteristics of native PEI have also been reported.⁴³ As expected, the chemical modification of PEI affected its proton sponge capacity, hydrophobic–hydrophilic balance, lipophilicity, and cytotoxicity and transfection efficiency.

Owing to the polybasic activity of poly-L-histidine, some poly-L-histidine polymer-conjugated polymers are also introduced and tested for application to polymeric gene carrier systems.^{44,45} Putnam and coworkers have reported that when histidine residue was conjugated to the backbone of the linear poly-L-lysine polymer, the grafted copolymers showed remarkably enhanced gene expression compared to native poly-L-lysine.⁴⁶

As described above, one of the major problems with nonviral gene delivery systems is lower efficiency compared to viral vectors. Many techniques have so far been attempted to overcome such problems by linking or conjugating cell-specific ligands and TAT-derived peptide or oligopeptide, such as oligoarginine derivatives. Recently, some basic peptides known as protein transduction domains (PTD) or membrane translocalization signals (MTS) were identified, characterized, and used for delivery of drugs, proteins, oligonucleotides, and plasmid DNA.^{47,48} These peptide sequences usually contain positively charged amino acid residues, i.e., arginine and lysine. The mechanism of enhanced cellular uptake by these peptide molecules is still unclear and there is debate about whether their entry into cell membranes follows an endocytic pathway or nonendocytic pathway or direct penetration into membranes.⁴⁹ While most experiments were usually performed by covalently linking these peptides to polymers or lipids for nucleic acid delivery, they have also been simply mixed with nucleic acids for electrostatic interaction.

Arginine-oligopeptides modified with several hydrophobic lipids have been recently reported to be effective gene carriers and, interestingly, those peptides alone did not show a high level of transfection efficacy.⁵⁰ In addition, TAT-PEG-PE liposomal systems encapsulating plasmid DNA have been reported to be efficiently incorporated into cells in vitro and *in vivo*.^{51,52} The common characteristic of those systems is thought to be that the arginineresidues are rich on the surface of multi-valent liposomal systems. In addition to these reports, Okuda and colleagues recently reported that the arginine residues located on the surface of poly-L-lysine dendrimer exerted a pronounced influence on the transfection efficiency of the polymer.⁵³ It was also reported that branched-chain arginine peptides showed a different cellular localization, implying that a liner structure was not necessary, and the formation of a cluster of arginines was suggested to be important for translocation.⁴⁹

22.3.4 FABRICATION OF METAL SURFACE WITH CATIONIC MOIETY FOR DNA BINDING

In line with the development of integrated biological sensors, microelectroniccompatible materials such as silicon, glass, and gold can be physically and chemically modified for many applications. Yang and his coworkers reported that DNA could be efficiently and stably conjugated onto the surface of nanocrystalline diamond thin films by a photochemical modification method.⁵⁴ They also demonstrated that DNA-modified ultra-nanocrystalline diamond films showed very high stability and sensitivity compared to other commonly used surfaces, i.e., gold, silicon, glass, and glassy carbon. It is thought that the report provides a basic important principle for the development of injectable medical devices for gene delivery systems as well as drug delivery systems.

Another focus of the design of polymer/metal nanoparticle-mediated DNA delivery is the fabrication of surfaces of gold nanoparticles⁵⁵ and bimetallic nanorods (gold-titanium).⁵⁶ The use of metal particles for delivery of large-sized plasmid DNA through simple chemical modification of the surfaces was successfully implemented. Such technology involving polymer particles

containing metal may open up a new field of polymer-metal hybrid systems for gene delivery. However, the safety problem resulting from the use of metal nanoparticles remains to be thoroughly studied for further clinical application.

22.3.5 LIGANDS FOR TARGETING

Traditional polycationic charge-based polymers are reported to form complexes with DNA displaying high cationic surface charge. Such surplus surface cationic charge might contribute to increased gene expression for in vitro applications. However, there are many barriers for *in vivo* applications as reported by many groups, and the positively charged particles could not execute active targeting to some specific target cells or organs. In addition, within minutes after injection into the blood vessel, the serum components adsorb to the particulates regardless of their initial surface charge.⁵⁷

One simple approach to receptor-mediated transfection is to make use of mono- or disaccharides that are recognized and internalized by the asialogly-coprotein receptors of hepatocytes.^{58–60} The conjugation of the ligands to PLL in an appropriate configuration caused increased transfection of target cells in vitro and *in vivo*. Another attempt was to conjugate chemically the synthesized tripeptide Arg-Gly-Asp (RGD), which shows specific interaction with α V- and α 5 integrins.⁶¹ It was found that the introduced RGD sequence increased and affected the specific targeting and expression of the DNA complexes.

Hood et al. have developed a targeted gene delivery model that was efficient in *in vivo* experiments.⁶² These authors found the potent $\alpha v\beta 3$ -targeting ligand candidate from the library of small organic molecules and conjugated the ligand to cationic polymerized lipid nanoparticles for a site-specific gene delivery *in vivo*. There was pronounced tumor regression in mice after systemic administration of an antiangiogenic gene through specific targeting to the tumor vasculature.

Another approach for targeting cancer cells is to make use of folic acid because the folic acid receptor is generally over-expressed in many cancer cells, especially in ovarian carcinomas.⁶³ Therefore, many groups have tried to make the ligands tethered onto the surface of polymer/DNA complexes and, more desirably, situated at the end of polymer-conjugated PEG chains for anticancer drug delivery^{64–68} or gene delivery.^{32,69–74} In general, the polymers used to make folate-decorated polyplexes were pLL, PEI, or poly(dimethylamino-methylmethacrylate (pDMAEMA).

Rebuffat and coworkers have reported an interesting strategy for enhanced gene delivery and expression.⁷⁵ They devised a steroid-mediated gene delivery system, which makes use of the interaction between glucocorticoid and glucocorticoid receptors (GRs) that are located in the cytosol. So, once the steroid-decorated DNA is introduced inside cells, it is subsequently transported efficiently to the nucleus by high affinity receptor interaction. They suggested that nuclear receptors in the cytoplasm could be utilized as intracellular gene

delivery vehicles. In summary, efficient gene delivery polymers should be equipped with such reagents that facilitate nucleus uptake as well as the specific cell surface targeting ligands.

22.4 POLYMERIC NANOPARTICLE- OR INJECTABLE DEPOT-MEDIATED GENE DELIVERY

In addition to the polycationic polymers, physical DNA-trapping methods using degradable polymers have been studied.^{76–80} Many kinds of synthetic or natural polymers that are degradable are introduced for controlled release of DNA systems, which usually encompass either micro-, nano-particulate systems or DNA-releasing injectable matrix systems. Such particulate systems are generally produced by a double emulsion-solvent evaporation technique (water-in-oil-in-water system). Due to the degradation of the ester bonds, the DNA trapped inside is released slowly out of the particles. In these systems, sustained and regulated DNA release can be engineered by controlling many factors, and subsequent sustained gene expression may be applicable to the treatment of certain types of localized disease conditions. Compared to traditional charge-based polymers, the particles produced by this system show negative zeta potential values.⁸¹ This may prevent the particles from interacting with the extracellular matrix components, such as proteoglycans, after their in vivo administration.

By virtue of the development and accumulated knowledge of sustained release technology in drug delivery systems, it is believed that such technology could be successfully applied to gene therapy, compensating for some of the problems associated with polymeric gene delivery systems. For example, such a DNA-releasing depot system could consist of injectable or implantable matrix types, and nano- or micro-particulate formulation types for either localized or targeted gene delivery to the tissue of interest.

Collagen are generally used in clinical settings for tissue reconstruction and have gained the attention of some researchers for their possible utility as an implantable matrix and gene releasing system.^{82,83} Further applications have recently been reported as comprising interesting hybrid models for sustained DNA delivery systems composed of collagen loaded with DNA only or DNA with nonviral vectors.^{84–90} They are promising attempts and interesting models which combine the merits of different approaches, i.e., nonviral vector systems, biomaterial-based tissue engineering, and controlled drug release systems.

22.5 CONCLUDING REMARKS

Virus-mediated gene therapy systems have revealed more unpredictable risks than hitherto known as investigation has intensified, even though their efficiency is still far greater than nonviral vector systems. So, there is no doubt that the demand for safe, efficient and reliable nonviral vectors will continue to increase in contrast to viral vectors in the future. The birth of perfect magic nonviral vectors requires multidisciplinary scientific cooperation and research. Moreover, in line with the development of nonviral vectors that are equipped with multifunctionalities, such as specific targeting, high efficiency, degradability and nontoxicity, research directed toward enhancing knowledge of DNA- or RNA-based therapeutic gene design should also be helpful in setting up essential and promising gene therapy systems for treating certain types of human diseases that would otherwise be fatal.^{3,91–93}

Despite recent success in the development of novel nonviral gene delivery systems, there are still many barriers and hurdles to overcome even to the extent that some people still have a pessimistic view of gene therapy and refuse to admit its immense potential and practicality. However, with increasing knowledge of molecular biology, chemistry, biochemistry, pharmaceutics, bioengineering, and medicinal technology, it may no longer be impossible to use genes as therapeutics.

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