Handbook of Polymers for Pharmaceutical Technologies

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Handbook of Polymers for Pharmaceutical Technologies

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To my parents and teachers who helped me become what I am today.

Vijay Kumar Thakur

Contents

Preface

Polymers and their derivative materials have been used most frequently as attractive alternatives to traditional medicinal materials for a number of pharmaceutical applications. For instance, polymeric matrices are the most widely employed system for preparation of controlled release pharmaceutical dosage forms due to their simple and low-cost manufacturing process. There are three main types of pharmaceutical matrices: inert, hydrophilic and lipidic matrices, hydrophilic and inert matrices being the ones most employed. Different types of polymeric materials, namely ethylcellulose, polymethacrylates, polyvinyl acetate mixtures, cellulose ethers, hydroxypropylmethylcellulose, methylcellulose, sodium carboxymethylcellulose, chitosan, gums, xanthan gum, guar gum, polyethyleneoxide, sodium alginate and starch derivatives, to name but a few, have been used as materials for pharmaceutical matrices. Along with polymers, biomaterials with specific abilities to interact with biological structures have been widely used in the development of different devices intended for biomedical applications. Within this field, the interest in endogenous polymers as components of delivery systems has also gained increasing attention. The pharmaceutical role of polymers in the context of modern biomedical applications is increasing rapidly, such as in the form of macromolecular polymers to modify drug release. There are a number of different macromolecular polymers that can be used to modify drug release from Extended release (ER) matrices. The ER oral dosage forms provide a number of therapeutic benefits (i.e., improved efficacy, reduced frequency of administration and better patient compliance) and retain market share. Due to the costs involved in discovering, developing and testing their safety, and getting approval for new polymeric materials, a new focus has been directed towards the investigation of the use of pharmaceutically approved polymer blends as matrix formers. Combining polymers of different chemistries or viscosities is being studied extensively as a means of achieving and optimizing extended drug release from hydrophilic matrices. Chemical conjugation with other pharmaceutical polymers such as poly(ethylene glycol) (PEG) has also evolved into a well-established technology used to improve the physicochemical, biomedical and pharmacological properties of several therapeutic molecules, such as peptides, proteins, antibodies, antibody fragments, oligonucleotides, and small drugs. Ubiquitously present nucleophillic groups, such as the terminal -NH_2 group, the ε-NH_2 group of lysine and the -SH group of cysteine, have all been used to couple peptides and proteins to mPEG derivatives. Moreover, site-selective, reversible and enzymatic PEGylation have recently gained increasing attention among the biopharmaceutical community.

Different research efforts all around the globe are continuing to explore and improve the properties of these polymers. Researchers are collectively focusing their efforts on the inherent advantages of pharmaceutical polymers for use in their targeted applications. This book is solely focused on the structure and chemistry of pharmaceutical polymers. Some of the important topics include but are not limited to: polymeric materials for culturing

human pluripotent stem cells; polymeric systems in quick dissolving oral films; gellan – a pharmaceutical excipient; critical points and phase transitions in polymeric matrices for controlled drug release; use of various polymer combinations in extended release matrices; biomaterial design for human ESCsand iPSCs on feeder-free culture toward pharmaceutical usage of stem cells; new perspectives on herbal nanomedicine; endogenous polymers as biomaterials for nanoparticulate gene therapy; molecularly imprinted polymers as biomimetic molecules – synthesis and pharmaceutical applications; biobased pharmaceutical polymer nanocomposite – synthesis and chemistry; improving matters of the heart – use of select pharmaceutical polymers in cardiovascular intervention; polymeric prosthetic systems for site-specific drug administration – physical and chemical properties; prospects of guar gum and its derivatives as biomaterials; polymers for peptide/protein drugs delivery; etc.

Several critical issues and suggestions for future work are comprehensively discussed in this book with the hope that it will provide a deep insight into the state-of-art of pharmaceutical polymers. We would like to thank the publisher and Martin Scrivener for the invaluable help in the organization of the editing process. Finally, we would like to thank our parents for their continuous encouragement and support.

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Gellan as Novel Pharmaceutical Excipient

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Abstract

An excipient provides an effective therapeutic way for convenient and precise dispensation of medicine(s)/drug(s) to the desired site in order to achieve a long-lasting outcome during the period of treatment. Therefore, the techniques for delivering the drugs over a prolonged period of time, with a sustained release profile, have been constantly investigated. This article endeavors to provide an insight about the structural and physiochemical properties of gellan, with the intention of exploring the biological applications of gellan in the pharmaceutical sector. Gellan is a natural linear anionic natural polysaccharide which is commonly used in the food and cosmetic industries. The biodegradability, nontoxicity and wide applicability of gellan make it a suitable candidate for the pharmaceutical industry. The gellan excipients alone or in combination with other biopolymers have been investigated for a wide range of biopharmaceutical applications such as mucoadhesion, granulation, gene therapy and wound healing. Recent applications of gellan include its usage as pharmaceutical excipient in ophthalmic, nasal, buccal, periodontal, gastrointestinal, colon-targeted and vaginal drug delivery. Gellan has also been proven as a potential candidate for tablet coatings in order to produce a sustained release dosage system with improved drug dissolution.

Keywords: Gellan, mucoadhesion, microcapsules, nanoparticles, nanohydrogels, drug delivery

1.1 Introduction

An excipient is an inactive substance that is used along with the active agent or medicine(s) in order to provide a convenient and precise dispensation of it from the designed dosage formulations. Conventionally, excipients were only used as vehicles for giving the required weight and volume for the appropriate administration of the active ingredient, i.e., drug [1]. Whereas, the pharmaceutical role of excipients in a modern context is defined as dosage forms which play multifunctional roles such as enhanced drug stability, drug solubility/absorption, bioavailability and sustained release performance for better acceptability in patients. However, despite all these claims, a meticulous knowledge about the physical and chemical properties as well as information regarding the safety, management and regulatory status of the excipient materials are

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crucial, as they can no longer be totally considered as inactive ingredients. Thus, the design of novel and effective drug delivery systems has given rise to an increased number of excipients that are based on natural polymers.

The growing applications of natural polymers in pharmaceutical industry mainly relies on their abundance in nature, biodegradability, non-toxicity and their cost effectiveness as compared to synthetic polymers [2].

Gellan is a natural biocompatible polysaccharide which is obtained as a fermentative product from a pure culture of nonpathogenic microbial strain [3,4]. It has been successfully employed in solid, liquid and semi-solid dosage formulations. It has found enormous applications as gelling agent, thickening agent, stabilizer and foaming agent, which are precisely useful in the designing of improved drug delivery systems [5]. Gellan does not affect the chemical structure of formulated drug and get degraded by natural biological processes within the body. These properties of gellan circumvent the need for removal of the drug delivery system from the body after its action has been accomplished. Additionally, as an excipient, it helps to maintain a steady-state plasma concentration of drug at the desired site during the entire period of treatment, and also reduces the adverse effects of the drug by releasing the drug in a well-controlled manner. As compared to other polysaccharides, gellan exhibits better thermal stability, acid reliability, adjustable gel elasticity and high transparency, and is therefore a preferred candidate for the food and pharmaceutical industries [6]. Here, in this article, our emphasis is on gellan-based materials, and their chemical modification, with the intention of exploring the biological applications of gellan as pharmaceutical formulations such as drug release modernizers, gelling agents, implants, films, beads, microparticles, nanoparticles, injectable systems and granulating systems, as well as mucoadhesive formulations.

1.2 Structural Properties of Gellan

The purpose of analyzing the structural features of gellan is to understand the influence of chemical modifications on its physiochemical properties. Structurally, gellan is a linear, anionic polymer which is composed of tetrasaccharide repeating-units, comprising two molecules of monosaccharide β-D-glucose, one molecule of β-D-glucoronic acid and one molecule of α-L-rhamnose linked together in a linear fashion [4]. The percentage of the three main constituents of gellan is reported as approximately 60% for glucose, 20% for rhamnose and 20% for glucuronic acid [6]. The native form of gellan is found to be esterified with L-glycerate and O-acetate at 2 and 6 positions of the D-glucose. However, the commercially available Gelrite® is the de-esterified form of gellan [7].

The detailed structural analysis of gellan has been performed using X-ray diffraction technique by Chandrasekaran and Radha. The X-ray diffraction study on Li^+ gellan revealed that it possesses an extended double helical molecular structure formed by intertwining of threefold left-handed helical chains of pitch 56.4 Å in a parallel fashion [8]. This helical structure is stabilized by means of interchain hydrogen bond between the hydroxymethyl groups of 4-linked glucosyl units in one chain and carboxylate

group in the other chain [9]. The X-ray diffraction analysis of the K^+ salt also showed the K^+ ion is linked with the carboxylate group of gellan and surrounded by six ligands to attain a strongly anchored octahedral coordination which is responsible for the stability of double helical gellan structure.

Recently, atomic force microscopy (AFM) and dynamic viscoelasticity measurements have been employed for investigating the detailed chemical structure of Na^+-gellan. The study revealed that gellan is composed of a continuous network of structures that is mainly developed through the inter-helical associations of end-to-end type rather than the associations of side-by-side type. The presence of cations (K^+ ions) is found to be a necessary component for the development of these types of continuous network structures. The study further confirmed the fibrous model of gellan gelation instead of the conventional model which presumed that joining of the adjacent junction zones leads to formation of disordered flexible polymer chains [10,11].

On the basis of o-acetyl substitution of the polysaccharide chain, gellan can be categorized into two basic forms: (i) high acyl form and (ii) low acyl form (Figure 1.1). Both forms exhibit different characteristic properties (Table 1.1). The acyl substitution of gellan chain shows an intense effect on its gelling characteristics. The high acyl form of gellan produces soft, elastic and non-brittle gels, whereas the low acyl form yielded steady, non-elastic and brittle gels [12].

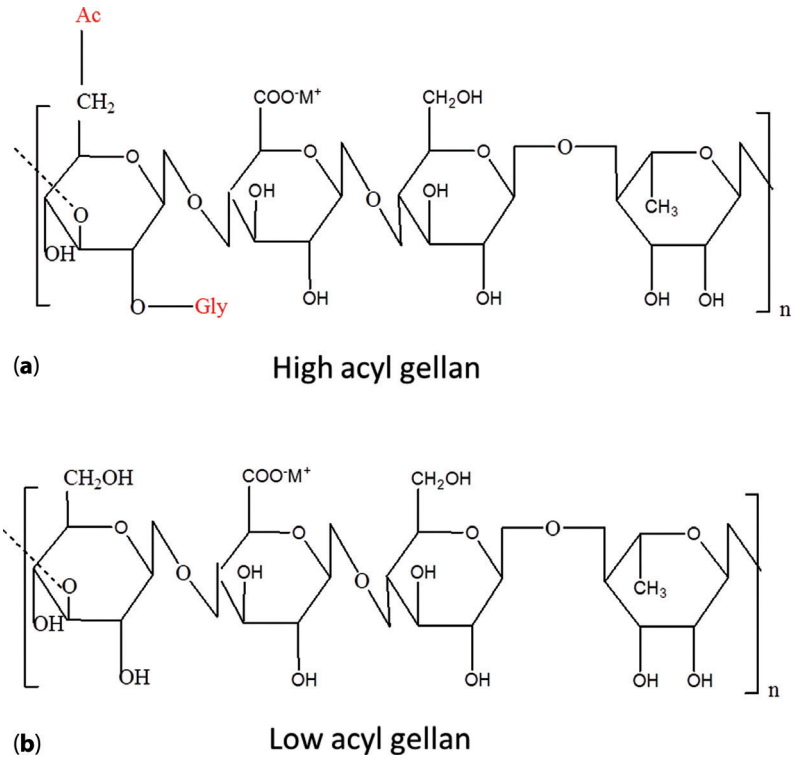

Figure 1.1 Chemical structure of repeating units of gellan.

Table 1.1 Comparison of the physical properties of high-acyl and low-acyl gellan.

	High Acyl Gellan	**Low Acyl Gellan**
Molecular Weight	$1–2 \times 10^6$ Daltons	$2–3 \times 10^5$ Daltons
Solubility	Hot water	Hot/ or cold water
Setting Temperature	70°–80°C (158°–176°F)	30°–50°C (86°–122°F)
Thermo-reversibility	Thermo-reversible	Thermo stable

1.3 Physiochemical Properties of Gellan

Gellan exhibits high gel strength, an excellent stability, process flexibility and tolerance, high clarity and an outstanding active reagent release property. The physiochemical properties of gellan are listed in Table 1.2 [13–15].

1.3.1 Gelling Features and Texture Properties

Gellan is a polysaccharide which has a characteristic property of temperature-dependent and cation-induced gelation. Gellan abruptly undergo sol-gel transitions (phase transition) and forms gels on heating and cooling of its solutions in the presence of cations (Figure 1.2). These gels are transparent and resistant to a wide range of heat and pH [16]. Light scattering analysis demonstrated that the gelation behavior of gellan involves two separate thermo-reversible steps: (i) at low temperature/or on cooling gellan converted in an ordered double helix from two single chains, and (ii) on high temperature/or heating it changes from a double helix to two single-stranded polysaccharide chains [17,18]. Rheological studies have indicated that at low temperature, gelling of gellan occurs due to the coil-to-helix transition [19]. The mechanism lying behind the gelation of gellan includes the synthesis of junctions in double-helix and the aggregation of these junctions to develop a three-dimensional network in the presence of cations and water [20]. Since gellan is a polyelectrolyte, the presence of divalent or monovalent cations markedly influences the texture and properties of gellan gel. The presence of cations has always been found to promote the gelation process by producing a shelding effect over the helices, thereby enhancing the probability of hydrogen bond formation by inter-helical interaction. One of the mechanisms explain by Gunning *et al.* showed the development of distinct junction zones on the helical polymer chains and the presence of mutual interactions (inter-helical associations) between the connecting adjacent junction zones [21]. These junction zones are thought to be stabilized by cation binding. The addition of salt solution significantly increases the number of bridges at a junction zone, and thus improves the elastic modulus and gelling properties of gellan. Another investigation explaining the gelation process in gellan has been accomplished using atomic force microscopy (AFM) technique. It supports the fibrous model of gellan gelation, which states that in the presence of cations,

Table 1.2 Physiochemical properties of gellan.

Color	**Off White**
Molecular weight	>70,000 with 95% above 500 000
Bulk density	Approximately 836 kg/m3
Solubility	in water; insoluble in ethanol
pH (1% solution)	Neutral
Gel strength	≥850 g/cm2
Specific gravity	<1
Stability	Stable at room temperature
Moisture content	98.6%wb or 67.6% db
Loss on drying	≤15.0% (105°, 2½h)
Nitrogen	≤3%
Isopropyl alcohol	≤750 mg/kg
Microbiological criteria	Bacterium count: ≤10,000 cfu/g *E. coli*: Negative by test Salmonella: Negative by test Yeasts and molds: ≤400 cfu/g

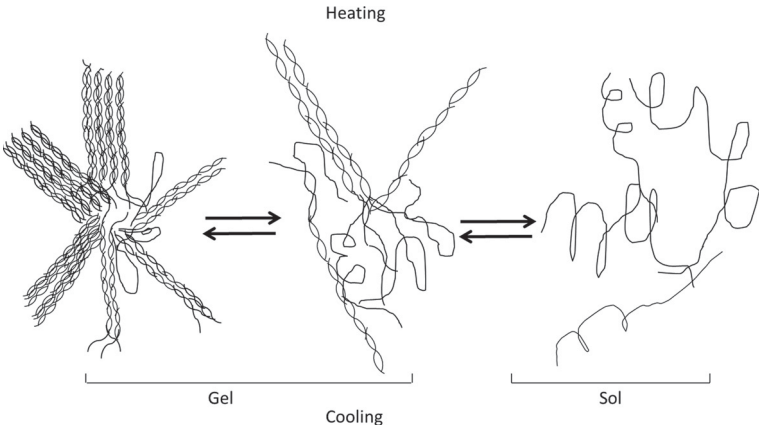

Figure 1.2 Schematic model representing the gelation mechanism for gellan.

some primary fibers form during the process of coil to helix transition. This process is followed by the aggregation of primary fibers into thicker branched fibers which results in the enhanced elastic behavior [22].

1.3.2 Rheology

Rheological assessments are appropriate tools in order to attain the organizational information about the macromolecules in a certain medium. The rheological properties of gellan solutions in the presence and absence of salt were reported by Miyoshi *et al.* [19]. They evaluated the steady shear viscosities and oscillatory measurements for gellan solution. The data obtained from this study suggested that in the absence of salt, gellan solution followed a shear-thinning behavior and its conformation changes from a compact coiled structure to a helical structure (the helical structure compared to coiled structural conformation can be more easily oriented with the shear flow). At low shear rates, the range of Newtonian plateau was found to become gradually narrower because of the development of an ordered structure of gellan in the solution**.** Whereas, in the presence of a sufficient concentration of cations, gellan associates in highly ordered structure and tends to form weak gel, which significantly followed a shear-thinning behavior with no Newtonian plateau even at a relatively high temperature [23].

In another investigation, the rheological properties of gellan solution were assessed on the basis of o-acetyl substitution of the gellan chain. The studies carried out with chemically modified gellan suggested that the rheology and conformation of gellan is directly influenced by the level of acetate and glycerate molecular substitution present in its chains. However, the data obtained from X-ray fiber diffraction molecular analysis, proposed that glycerate alone is an important factor that determines the association of gellan chain as well as its rheological properties [24].

Rodríguez-Hernández discussed the rheological properties of gellan polymer in terms of the visualized microstructure. Confocal laser scanning microscopy (CLSM) technique has been opted for the imaging of the gellan aqueous systems with their rheological behavior in order to identify the extent of chain associations. The microscopic observations suggested the formation of three-dimensional (3D) networks of gellan in its aqueous solution rather than gellan aggregates [25].

1.3.3 Biosafety and Toxicological Studies

Toxicological studies revealed that gellan is relatively nontoxic to animals when administered as a single large dose (LD_{50} = 5000 mg/kg) in the diet (Table 1.3), while an inhalation toxicity test found it caused no deaths in a group of 10 animals [6]. An eye irritation test described in the above study confirmed the safety of gellan in the case of contact with eyes. Gellan formulations received their first approval for food applications in Japan in 1988. It is now acceptable for food, non-food, cosmetic and pharmaceutical use in the United States, Canada, Australia, Latin America, South America, Asia, and in the European Union [12].

Table 1.3 Acute toxicity of gellan.

Species	Sex	Route	LD50	Reference(s)
Rat	Male & Female Male & Female	Oral Inhalation	>5000mg/kg >5.09 mg/l	[26] [27] [28]

1.4 Pharmaceutical Applications of Gellan

1.4.1 Gellan-Based Pharmaceutical Formulations

1.4.1.1 Gel Formulations

Gellan is capable of forming gels in the presence of counterions. These gels are predominantly strong when formed in the vicinity of divalent ions as compared to monovalent ions [29]. Recently, the suitability and acceptability of gellan hydrogels in the area of regenerative medicine and drug delivery were studied by Correia *et al.* They invented gellan comprising photocrosslinked hydrogelic formulation which was able to control the encapsulation and reticulation of animal cells and/or drugs, or their combinations [30]. Similarly, a gellan-based floating *in-situ* gelling system has been developed for controlled drug delivery of amoxicillin by Rajinikanth *et al.* They observed that the floating *in-situ* gels containing ten times lesser amount of amoxicillin than the amoxicillin amount in solution form were more effective for treating an infection caused by *Helicobacter pylori* [31,32].

1.4.1.2 Mucoadhesive Formulations

Mucus is a viscous and slippery secretion that covers many epithelial surfaces of the body. The mucus secreting cells are extensively found in different areas of the body, such as in the nasal, ocular, buccal, gastrointestinal, reproductive and respiratory areas. Gellan can potentially be used as mucoadhesive drug delivery systems, as it is a water-soluble polymer which becomes adhesive when it comes in contact with mucous membranes, and subsequently provides protection to the encapsulated drug from enzymatic degradation. This property of gellan also increases the contact/residence time of encapsulated drug [33]. Hence to investigate the mucoadhesive properties of gellan, Ahuja *et al.* developed modified bioadhesive carboxymethyl gellan gel beads as drug delivery vehicles [34]. The comparative evaluation of modified carboxymethyl gellan showed 2.71-fold higher mucoadhesive strength than the gellan alone. The outcomes showed that the carboxymethylation modification of gellan improved its mucoadhesive properties and hence augmented its aqueous solubility and gelling behavior. They reported that carboxymethylated gellan gum does not gel at 0°C even at a concentration of 10% (w/v). Their observations revealed 100% bioadhesion of metformin (model drug) containing ionotropically gelled beads over a period of 24 h. Further, it was concluded that carboxymethyl gellan beads released the model drug metformin at a more rapid rate than gellan alone [34].

Viram and Lumbhani developed gellan containing mucoadhesive *in-situ* gels for the controlled release of the drug metoclopramide hydrochloride [35]. They showed that the release kinetics of gellan formulation rely on the diffusion model for drug delivery and the release rate of drug was strongly dependent on the weight fraction of the gellan in the formulated tablets. The *in-vitro* mucoadhesion studies and drug release profiles showed that the increasing gellan concentration was accompanied with a slower drug release rate as well as favorable retention of gellan gel in nasal mucosal tissue. Hence, it improved the drug absorption at the target site [36].

1.4.1.3 Granulating/Adhesive Agents and Tablet Binders

Granulating agents or binders provide stability to the tablets which is required by them during their processing, handling, packaging and transportation, as well as also improving compressibility and fluidity of drug powder [37]. The micro-mimetic studies revealed that microwave-assisted physical modifications can improve the efficacy as well as proficiencies of the gellan-based drug excipient [38,39]. Gellan has also been proved as an efficient and outstanding granulating agent as compared to earlier reported gelatin and maize starch for chloroquine phosphate tablets [40].

1.4.1.4 Controlled Release Dosage Form

The easy-to-swallow controlled release solid dosage forms (gels, films, coatings, tablets, etc.) can be simply produced by using gellan. Yang *et al.* explored the formulation having polyelectrolyte complexes of cationic polymer chitosan and anionic polymer gellan for controlled release of proteins. They reported that the higher gellan concentration in the prepared formulations significantly retarded the fast release of protein and achieved sustained release. The protein release behavior mainly followed the Fickian diffusion mechanism [41]. Similarly, alkaline phosphatase (ALP) encapsulated capsules of gellan-chitosan hybrid have been synthesized using polyionic complex reactions occurring between the oppositely charged polysaccharides. These polyionic complex capsules can potentially be employed in the pharmaceutical industry, as these complexes are biodegradable and biocompatible, can be implanted directly into the organisms, eliminate the need for surgical removal of formulations after use, and show bioresorbability [42,43]. The crosslinked gellan hydrogels for controlled and modified drug release of high molecular weight bioactive molecules such as proteins (Vitamin B12, fluorescein isothiocyanate-dextran), were prepared and characterize by Matricardi *et al.* [44,45]. Crosslinking significantly enhanced the mechanical properties of gellan hydrogels and slowed down the release profiles of drug. The same phenomenon was further confirmed by the studies of Mangond *et al.,* who proposed crosslinked gellan microbeads loaded with ketoprofen as a sustained drug release system. The drug release profiles of ketoprofen from gellan microbeads were found to follow non-Fickian mechanism [46]. A complex formulation of gellan and calcium was developed for the sustained oral administration of paracetamol drug. This formulation caused the formation of gellan gels in the stomachs of animals (rabbits and rats) and provided controlled drug release over a period of 6 h [47]. The gellan hydrogels were also modified physically and chemically in order to provide improved drug entrapment [44]. The differences between the two types of modified gels were evaluated with respect to their ability of retention for the model drug $DexFluo_{70}$ (fluorescein isothiocyanate-dextran). Chivers and Mooris reported the theophylline drug containing gellan gels as sustained release commercial liquid dosage forms and their evaluation of the bioavailability of the drug within the gels was found to increase by 4-5-fold in rats and 3-fold in rabbits compared to a commercial sustained release liquid dosage form [48,49].

1.4.1.5 Microspheres and Microcapsules

Gellan polyionic microspheres have also been tested for the encapsulation of biological components. The use of capsules and microspheres not only offers sustained release, but

also provides the protection of encapsulated substances. The small particle size of these formulated microspheres enables an easy administration of bioactive molecules either by oral route or by injection [50]. A recent investigation briefed the self-destructing "mothership" capsules for timed release of encapsulated bioactive contents (an enzyme chitosanase). These capsules were designed via single-step assembly which causes self-destruction at a later time because of their packaged enzymatic contents (chitosanase is capable of degrading chitosan polymer into small oligomers). The capsules were formed by taking the benefit of electrostatic interaction between the anionic polymer, gellan and cationic polymer, chitosan. The fabricated capsules were called "motherships" and were engineered to transport the small cargo molecules termed as "babyships" [51]. Similarly, aceclofenac-loaded capsules comprised of alginate and gellan were developed for prolonged drug release using maleic anhydride-induced unsaturated esterification method [52]. The *in-vitro* drug dissolution profiles of aceclofenac confirmed the controlled release of it by following the Korsmeyer-Peppas model. The results of *in-vivo* studies in which the aceclofenac drug is orally administrated in rabbits, demonstrated the prolonged systemic absorption of drug. Novel gellan-poly (N-isopropylacrylamide) thermoresponsive semi-interpenetrating microspheres using ionic crosslinking method have been explored by the research group of Mundargi *et al.* They exploited the developed microspheres for controlled release of atenolol, an antihypertensive drug. The *in-vitro* drug release profiles indicated the temperature dependency of drug release, which was found to be extended up to 12 h [53]. Another type of interpenetrating polymeric (IPN) microspheres comprised of complex of gellan and poly(vinyl alcohol) have been synthesized by emulsion crosslinking method for sustained release of carvedilol [54]. Microcapsules encompassing oil and other core materials have also been synthesized using a complex coacervation of gellan and gelatin biopolymers [48].

1.4.1.6 Gellan Beads

Recently Narkar *et al.* formulated an amoxicillin drug containing mucoadhesive gellan beads. The beads were fabricated via ion-induced gelation method, which were then stabilized using acidic and alkaline cross-linking media. The beads were subsequently coated with another biopolymer (chitosan) in order to achieve controlled drug release. These chitosan-coated gellan beads displayed controlled *in-vitro* drug release up to 7 h [55]. In another embodiment, a gellan hydrocolloid bead formulation containing diltiazem hydrochloride was produced as potential drug vehicle [56]. These beads were comprised of several fillers such as talc, kaolin, calcium carbonate, potato or corn starch (10%, w/w). The study demonstrated that the drug loaded gellan beads undergoes swelling after coming in contact with simulated gastric fluid and intestinal fluid. The filler inclusion proposed in this study was found to enhance the stability of gellan beads and subsequently prolonged the time of drug release. Similarly, gellan beads encompassing cephalexin as a model drug were formulated by extruding the dispersive solution of gellan and cephalexin into a mixed solution of counterions (calcium and zinc ions). The morphology of beads and release rate were optimized by varying the process variables such as pH of counterion solution and cephalexin loading [57]. A rather simpler ionotropic gelation method to encapsulate a hydrophilic drug, propranolol hydrochloride, in gellan beads was opted for by Kedzierewicz *et al.* They fabricated the beads by first making a dispersion of drug and gellan and then dropping this

dispersion in an ionic solution of calcium chloride. It was observed that these gellan beads could be stored for up to three weeks in a wet or dried state without any alteration in drug discharge [58]. Development of gellan beads has also been experimented on to assess the effect of divalent cations on drug encapsulation efficiency. The hard gel beads prepared with different cations are shown to significantly affect the aqueous solubility of the drug. Furthermore, it was concluded that the electro-positivity of cations plays a significant role in the gelation of gellan and drug loading can be increased by using divalent ions of high atomic number [29,59].

1.4.1.7 Gellan Films

Xu *et al.* prepared dried antimicrobial films of gellan and konjac glucomannan using a solvent-casting technique with different blending ratios of the two polymers. In addition, the suitability of formulated films was evaluated for release of incorporated nisin, an antimicrobial drug. The films incorporated with nisin were found to have antimicrobial activity against *Staphylococcus aureus.* The conducted investigation demonstrated that by increasing the content of gellan, the antimicrobial effects of the films were also enhanced [60]. Correspondingly, gellan film as an implant for insulin delivery was also developed as a candidate for maintaining blood glucose [61]. The study reported that the blood glucose levels of the diabetic rats implanted with insulin encompassed films were about half of those implanted with blank gellan films, and this therapeutic effect of insulin could last for one week. The conclusive *in-vitro* and *in-vivo* studies suggested that the developed gellan film could be an ideal candidate in the development of protein delivery systems.

1.4.1.8 Gellan Nanohydrogels

A novel nanohydrogel system (NH) using gellan biopolymer has been developed by the research group of D'Arrigo *et al.* These gellan nanohydrogels were designed to carry and deliver anti-cancer and anti-inflammatory drugs simultaneously. Paclitaxel (an anti-cancer drug) was physically entrapped in these fabricated nanohydrogels, whereas Prednisolone (an anti-inflammatory drug) was entrapped chemically with the carboxylic groups of gellan. Nanohydrogels acted to increase drug solubility as well as drug bioavailability and hence displayed an improved drug performance. Data suggested that the synergistic effect of the anti-inflammatory and anti-cancer drugs from the developed nanohydrogels favor an augmented *in-vitro* cytotoxic effect on cancer cells [62].

1.4.1.9 Gellan Nanoparticles

Gellan has been proved as a potential material for carriage of fragile drugs and, therefore, has been explored extensively to provide new opportunities in the field of drug delivery [63]. Palaniappan has formulated a controlled release nanoparticle composition comprised of gellan and polyethylene glycol polymers and exploited it for delivery of proteins or an anti-carcinogenic compound [64]. The nanoparticles have been further surface functionalized with bifunctional ligand in order to provide an affinity to the material and for targeted drug release. Dhar *et al.* introduce a new application

of gellan and suggested that gellan can be used as a reducing agent to synthesize gold nanoparticles with a greater stability toward electrolyte and pH changes. These nanoparticles have been assessed for controlled release of doxorubicin hydrochloride, an anthracycline ring antibiotic. The study concluded that the effectiveness of doxorubicin hydrochloride-loaded nanoparticles has an enhanced cytotoxic effect on human glioma cell lines NIH-3T3 and LN-229 [65].

1.4.2 Role of Gellan Excipients in Drug Delivery and Wound Healing

1.4.2.1 Ophthalmic Drug Delivery

An ideal ophthalmic formulation should be installed in the ocular area without causing any irritation or blurred vision [29]. The *in-situ* gel-forming drug delivery system is often considered as suitable ophthalmic formulation because after administration in the ocular area, it immediately undergoes phase transition to form viscoelastic gel, which in turn enhances the residence period of the drug at the target site to yield better drug performance [66].

Gellan has a characteristic property of cation induced *in-situ* gelation that can be used for sustained ophthalmic drug delivery applications [67,68]. These *in-situ* gel-forming systems could prolong the precorneal residence time of a drug and improve ocular bioavailability [69,70]. Recently, Timoptic-XE®, an ophthalmic drop formulation (Merck & Co., Inc., Whitehouse Station, NJ, U.S.) which comprises gellan, was introduced in the market. Its administration once a day is equally effective in lowering the intra-ocular pressure (IOP) as the equivalent concentration of simple eye drops of aqueous solution of timolol maleate (Timoptic®, Merck & Co., Inc.) administered twice a day [71,72].

Liu *et al.* developed an ion-activated *in-situ* gelling vehicle comprised of gellan (Gelrite) and alginate polymer solution for ophthalmic delivery of matrine drug [46]. They investigated the effect of the developed formulation on *in-vitro* and *in-vivo* precorneal drug release kinetic of matrine. *In-vitro* release and *in-vivo* pharmacological studies revealed that the Gelrite/alginate solution had an ability to improve drug retention compared with the Gelrite or alginate solution alone. The combination of Gelrite and alginate solutions significantly increased the gel strength under physiological conditions, and this combined solution was found easy to administer during ocular instillation. The tested formulation was found to be almost a nonirritant in the ocular irritancy test [73].

Gellan can be gelled in the tear fluid even at a very low polymer concentration. Due to this property, in physiological conditions where the formulated instilled drops are diluted, gellan can form gel with a high elastic modulus [74].

Balasubramaniam *et al.* successfully formulated indomethacin containing gellan-based *in-situ* gelling system as a viable alternative to conventional eye drops. These developed formulations provided sustained release of the drug in addition to prolonging the residence time in corneal region, thereby enhancing the ocular bioavailability. The formulated system did not cause any deleterious effects to the ocular tissues [75].

Sanzgiri *et al.* synthesized a methylprednisolone (MP) ester of gellan (gellan-MP) as a sustained release dosage form. They prepared two types of gellan films, one with physically incorporated MP and the other was suspended with MP. *In-vitro* drug release profile of MP from the test dosage forms was found to follow anomalous kinetics in the case of gellan films in which MP was physically incorporated, whereas gellan films suspended with MP released covalently bound MP in an approximate zero-order pattern. The *in-vivo* studies concluded that gellan-MP ester can be used to increase the residence time of methylprednisolone in the tear fluid of rabbits [68].

1.4.2.2 Nasal Drug Delivery

Gellan gum is a biodegradable and biocompatible polymer which does not cause any damaging effects in the nasal mucosal cavity even if persistently used for longer periods [76]. Shah *et al.* prepared gellan microspheres containing sildenafil citrate as a model drug using the spray drying method for intranasal delivery to avoid the first pass metabolism. Studies indicated the diffusion controlled delivery (Higuchi model) of drug from the gellan microspheres. In the nasal mucosa, gellan microspheres are supposed to form highly viscous gel by withdrawing water from the nasal mucosa and interacting with cations present in nasal secretions. The resultant gel formation decreases the cilliary clearance rate, and as a consequence, the residence time of the formulation at the nasal mucosa is prolonged [76]. In another investigation, gellan formulation containing fluorescein dextran as a model molecule has been tested *in vivo* for nasal drug delivery [69,59]. This gellan formulation initially behaves as a fluid but turns into a rigid gel when it comes in contact with the cations present in the nasal cavity [77]. Therefore, gellan formulations can be potentially used as nasal spray pumps due to its beneficial property of initial low viscosity succeeding to gelling upon contact with animal mucosa.

1.4.2.3 Oral Drug Delivery

Gellan has also been investigated for oral drug delivery [43]. Recently Yang *et al.* synthesized albumin integrated chitosan-calcium-gellan composite beads by a combination of ionotropic gelation and polyelectrolyte complexation methods for evaluating the controlled delivery of proteins to oral cavity [41]. The beads which were developed through polyelectrolyte amalgamation of chitosan and gellan not only reduced the burst release of albumin in simulated stomach fluid, but also prolonged the albumin residence time in the intestinal and colonic fluids. Similarly, the gellan-based formulation containing calcium ions and sodium ions complexes with a model drug theophylline has been prepared. The formulated gellan-drug system remained in its liquid form until it reached the stomach, and only released the drug in the highly acidic environment of the stomach. In the stomach environment, gelation occurred after a few minutes and persisted for several hours [29]. The *in-vitro* sustained release of theophylline from the developed gellan gels followed root-time kinetics over a period of 6 h. The results obtained from the above-described investigation concluded that the bioavailability of theophylline from gellan gels in the stomachs of animals was found to be increased by 4-5-fold in rats and 3-fold in rabbits, as compared to the commercially available oral formulations. There was no significant difference observed in the mean residence time of the theophylline drug in the stomach when administered through both types of vehicles [78].

1.4.2.4 Buccal Drug Delivery

The buccal mucosal environment refers to a more desirable choice for drug delivery if prolonged drug retention is required. This is due to less permeability of the buccal site than the sublingual site. It prevents the premature drug degradation and drug activity loss because of the harsh conditions present in the gastrointestinal tract (GI). In addition, drug can be injected, confined and removed easily after the treatment period from the buccal cavity [29]. An ideal buccal drug delivery system should halt in the oral cavity for some time and it should release the drug in a controlled manner using a unidirectional way [79]. Mucoadhesive polymers enhance the habitation time of the delivery vehicle in the oral cavity, and the double-layered structural design is supposed to provide the drug delivery in a unidirectional mode towards the mucosa and also circumvents the chance of loss of drug due to wash-out with saliva [80]. An effective, directly compressible Fluvastatin-containing buccal adhesive tablet with excellent bioadhesive force and good drug stability in human saliva has been proposed by Shah *et al.* They studied the mucoadhesive potential of gellan and reported that sustained drug release of Fluvastatin can be achieved by combining the gums (gellan) with other mucoadhesive polymers such as chitosan [81].

Remuñán-López *et al.* developed a new buccal bilayered device which is comprised of a drug along with a mucoadhesive layer and a drug-free backing layer. The formulated bilayered tablets were prepared by direct compression method. The mucoadhesive layer of the bilayered formulation was composed of a mixture of nifedipine and propranolol hydrochloride as model drug, chitosan and an anionic crosslinking polymer gellan. It was concluded that these developed devices can be used as promising candidates for controlled delivery of drugs to the buccal cavity [82].

1.4.2.5 Periodontal Drug Delivery

The local delivery of drugs and antimicrobial and other bioactive agents through a sustained release system into the periodontal pocket has received considerable attention in the active areas of pharmaceutical development and clinical research [83]**.** A gellan-based smart gel periodontal drug delivery system has been designed for local delivery of chemotherapeutic drug in the periodontal cavity [84]. The developed smart gel formulation consists of a model drug, ornidazole, along with gellan and lutrol F127 polymers. *In-vitro* drug release profiles showed that ornidazole release was significantly decreased with the increasing concentration of each polymer component in the formulated gellan smart gels. The delivery of antimicrobial therapy directly to the periodontal pockets has the significance of putting additional drugs at the target site, while minimizing the risk of exposure of the body to the drug [85].

1.4.2.6 Gastrointestinal Drug Delivery

The calcium chloride crosslinked gellan formulations were evaluated as a gastroretentive drug delivery system for controlled release of the drug ornidazole in order to treat gastric ulcers associated with *H. pylori* [86]. Their observations showed that the concentration of gellan in the prepared formulation significantly affected the *in-vitro* release profile of the drug. When these formulations were added to acidic or neutral media, they were found to become buoyant and provide better prospective controlled

drug release with enhanced gastric retention capability, which can effectively eradicate the *H. pylori* in order to cure peptic ulcer.

Floating raft formulations comprised of gellan as gelling polymer and verapamil hydrochloride as a model drug have been prepared. These formulations showed the advantage of liquid oral dosage form along with sustained drug release, and also prolonged the gastric retention period. They undergo pH-dependent sol-gel transition at gastric pH; hence prolonging the retention of the system in the stomach [87].

Doshi and Tank formulated dummy tablets of gellan using the wet granulation fabrication technique and discovered their feasibility as gastro-retentive tablets [88].

Gellan-chitosan polyelectrolyte complex beads, prepared by solution extruding method, have been explored for their potential application in delivery of metronidazole and metronidazole benzoate to the gastrointestinal tract [89].

A floating *in-situ* gelling system of clarithromycin (FIGC) using gellan as gelling polymer and calcium carbonate as floating agent has been designed by Rajinikanth and Mishra and potentially exploited for treating the gastric ulcers associated with *H. pylori* [90]. It was concluded that the floating *in-situ* gel of clarithromycin enhanced clarithromycin stability as well as increased the persistence of the drug in the gastrointestinal tract, which leads to complete clearance of *H. pylori* [90,91].

Foda and Ali summarized the potential applications of gellan as gastro-retentive drug delivery systems for enhancing the efficiency of antibiotics [32].

A gellan-based intragastric floating *in-situ* gel system for controlled delivery of amoxicillin has been investigated in order to treat peptic ulcer disease caused by *H. pylori*. These gels were found to be feasible for developing rigid gels when they come in contact with the gastric environment. Data has suggested that due to the prolonged gastrointestinal residence time, amoxicillin-containing gels were more efficient than that from the amoxicillin suspension for eradicating *H. pylori* from the gastrointestinal tract [31].

1.4.2.7 Vaginal Drug Delivery

The vaginal drug delivery system, unlike other drug delivery routes, offers many advantageous applications; it is a highly dynamic system with respect to absorption of drugs, their metabolism and their elimination. Vaginal targeted drug delivery systems also avoids hepatic first-pass metabolism, reduces the gastrointestinal as well as hepatic side effects, and also circumvents the chance of pain, tissue injury, and infection [92,93]. The vagina is a favorable site for systemic drug delivery as it has a large surface area, high permeability and very rich blood supply, but the prolonged retention of drug in the vaginal tract is often challenging due to the self-cleansing action [94]. The vaginal cavity has traditionally been used for local delivery of drugs such as prostaglandins, steroids, antibiotics, antifungals, antiprotozoals, antichlamydials, antivirals, and spermicidal agents [95,96].

Gupta *et al.* developed a chitosan/gellan gum-based *in-situ* gelling system for clindamycin drug delivery into the vaginal tract. The introduction of chitosan in the developed formulation improved the bioadhesive and permeation properties of the system, whereas gellan prolonged the retention time of clindamycin in the vaginal tract by forming an ion-activated gel immediately after coming in contact with vaginal fluid [97].

1.4.2.8 Colon Drug Delivery

Colon targeted drug delivery is greatly desirable for the treatment of a variety of bowel ailments [98,99]. An ideal colon drug delivery system should be proficient in protecting the drugs from premature degradation due to the chemical and therapeutic changes occurring in organs apart from colon. Colon mucosa shows low proteolytic activities, which makes it a suitable site for absorption of protein drugs [100,101]. Colon targeted drug delivery systems prevent the enzymatic degradation (held in the duodenum and jejunum) by releasing the drug directly into the ileum or colon, which in turn leads to superior systemic bioavailability [102,103].

The gellan beads containing azathioprine (AZA) drug have been formulated for colon-specific sustained drug delivery [104]. This study reported the biodegradability of gellan in the presence of galactomannanase for exploring its suitability for the development of colon-specific controlled delivery systems. Rheological studies proved that degradation of gellan due to the galactomannanase was concentration-dependent rather than time-dependent, which approved the feasibility of gellan as a drug carrier for sustained colonic delivery.

1.4.2.9 Wound Healing

Recently, Cerqueira *et al.* proposed a strategy that allows the self-organization of skin cells leading to improved healing. They formulated gellan gum/hyaluronic acid (GG-HA) spongy-like hydrogels, which were entrapped with the human dermal/epidermal cell fractions, and transplanted them into full-thickness mice wounds. The designed constructs led to rapid wound closure rate and were found to accelerate the wound healing process, re-epithelialization, as well as neo-tissue vascularization [105].

The films/xerogels comprising a cellulose ether, gellan and alpha-1-antichymotrypsin (ACT) have been invented as wound dressing material. These dry films/xerogels were designed to provide stable delivery of active ingredients that can be applicable in the fields of cosmetics and medicine. When applied to wounds, the formulated dry films get rehydrated immediately as they come in contact with the moist wounds, thus serving as a hydrogel/xerogel loaded with active ingredients (i.e., ACT) that is delivered/released to the wound site in a controlled manner [106].

A gellan containing sprayable composition for wound healing or repairing skin injuries has been examined. The viscosity of the described composition has been found to increase after its application on the wound site, and an immobile gel or an elastic gel of gellan is formed at the site of interest. The specific advantages of this designed formulation over other wound healing compositions is that it can be applied in a mobile state to give intimate contact with the wound, and it turns into immobile elastic gel immediately after it comes in contact with the wound as an adherent cohesive mass. This mechanism reduces the tendency for the mobile gel to run out of the wound due to the force of gravity [107].

In addition, protective and water-insoluble biodegradable films based on gellan have been prepared, characterized and evaluated for their effects on the wound healing process. The prepared films were further crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide crosslinker (EDC) to provide a optimum mechanical strength and make

them potentially suitable for biomedical applications such as wound healing and skin tissue replacement. EDC has the ablility to activate the galacturonic acid residues present within the gellan molecules. The *in-vitro* studies performed using MTT assay revealed that the gellan films are biocompatible with both the L929 fibroblast cells and blood cells. *In-vivo* studies further confirmed the bioactivity and the implementation of the formulated gellan films in clinical applications for accelerating wound healing [108].

1.5 Conclusion and Future Perspectives

The abundance of gellan in nature and its safe toxicological profile has encouraged researchers to explore its potential pharmaceutical and biomedical applications. The presence of carboxylic groups in gellan native chain is beneficial because of the modification of this unique polymer as well as the opportunity to react with several cationic drugs in order to design suitable polymeric drug delivery systems. The physiochemical characteristics of gellan such as gelation and mucoadhesive properties make it a promising candidate in drug delivery applications. Gellan may be used as a granulating material or tablet binder as a vehicle for peptide and gene delivery. In view of the abovementioned diverse pharmaceutical applications of gellan, it would be reasonable to say that these polymers have enormous potential for use as pharmaceutical adjuvants for conventional as well as for novel drug delivery systems.

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2

Application of Polymer Combinations in Extended Release Hydrophilic Matrices

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Abstract
Extended release (ER) oral dosage forms provide a number of therapeutic benefits (i.e., improved efficacy, reduced frequency of administration and better patient compliance) and retain market share. Due to the costs involved in discovering, developing, testing their safety and getting approval for new polymeric materials, a new focus has been directed towards the investigation of the use of pharmaceutically approved polymer blends as matrix formers. Combining polymers of different chemistries or viscosities has been studied extensively as a means of achieving and optimizing extended drug release from hydrophilic matrices. The present chapter will discuss the potential use of binary blends of various polymers to achieve the desirable release profiles.

Keywords: Hydrophilic polymers, polymer blend, swelling, drug release, matrices, compatibility, synergistic effect, ionic polymers

2.1 Extended Release Matrices

Among various medicinal dosage forms, tablets account for approximately 80% of the drug delivery systems used today due to their ease of manufacture, convenience of dosing and stability compared with liquid and semi-solid approaches [1].

The ER formulations provide therapeutic benefits such as improved efficacy and reduced side effects with reduced frequency of administration and, therefore, better patient compliance, and retain market share for the manufacturer [2–5]. Among ER formulations, matrix systems remain the most popular approach from the economics

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of development and manufacture as well as from the process control and scale-up points of view [1,6–9]. The most prevalent are hydrophilic matrices, which most often provide a desirable drug release profile, are cost effective and have a broad regulatory acceptance [5,10–12].

The majority of commercially available matrix formulations are in the form of tablets, and although developing them may initially seem simple, the formulation scientist is required to consider a number of variables that influence drug release, as well as the manufacturing and processing of these tablets. The release rate from the matrices is dependent upon drug characteristics; particle size, solubility and dose, release controlling polymers; type, level and particle size, fillers; type and level, tablet properties; porosity, tortuosity (affected by compression force) and shape [13–35].

2.1.1 Polymers Used in ER Matrices

There are a number of different macromolecular polymers that can be used to modify drug release from ER matrices. These can be classified into water soluble and water insoluble polymers. The full list of FDA-registered oral ER formulations containing commonly used hydrophilic or water-insoluble polymers together with their approved maximum potency levels are reported elsewhere [36].

2.1.2 Water-Soluble (Hydrophilic) Polymers

Amongst the various water-soluble or water-swellable polymers with high molecular weight used in hydrophilic matrices, hypromellose (hydroxypropylmethylcellulose [HPMC]) is the most commonly used polymer [5,13,37–40]. Other polymers have been studied and used on their own or in combination with HPMC to successfully modulate drug release. Examples include polyethylene oxide (PEO), with a recent review looking at its application in controlled release tablet systems [41]. Typical water-soluble hydrophilic polymers used may be classified based on their chemistry as follows:

- The cellulose derivatives of hydrophilic polymers including hydroxypropylmethyl cellulose (HPMC, hypromellose), methyl cellulose (MC), hydroxypropyl cellulose (HPC), hydroxyethyl cellulose (HEC) and sodium carboxymethylcellulose (Na CMC) [5,23,37,38,43–50];
- Non-cellulose derivatives including polymethacrylates, gums/polysaccharides such as sodium alginate (Na Alg), xanthan gum (XG), carrageenan, chitosan, guar gum (GG) and pectin [5,12,42,51–56];
- Vinyl polymers such as poly(vinyl alcohol) (PVA) and poly(vinyl pyrrolidone) (PVP) [42,44,57–62];
- Poly(alkelenes) like poly(ethylene oxide) (PEO) [5,42,63–70].

2.1.3 Water-Insoluble Polymers

Commonly used polymers for the development of inert matrices are ethyl cellulose (EC) [43,71–78], methacrylate amino ester copolymers and poly(butylmethacrylate) [79–83].

2.1.4 Fatty Acids/Alcohols/Waxes

To manufacture hydrophobic ER matrices the following materials are used: fatty acids such as carnauba wax, paraffin wax and cetyl alcohol [5].

2.2 Polymer Combinations Used in ER matrices

Due to the high costs involved in development and safety assessment (tox studies) of new polymers, to obtain acceptability in the market place, scientists use a combination of pharmaceutically approved polymers. This approach is to enhance the performance of the ER matrices as compared to single polymers. These are acheived by obtaining a variety of physical and chemical synergies between the polymers to enhance their properties in the formulation [84–87].

Polymer blends can be employed in the design of more robust formulations with more predictable *in vivo* release, i.e., less prone to dose dumping, reduce burst release; more resistant to media agitation (resembling food effect); with enhanced solubility and/or stability of some APIs [88–91]. Development and manufacture of ER matrices of freely water-soluble active substances, particularly where a high dose is used, is often challenging. This is due to a potential 'burst' release during the initial stages of dissolution, thus making the once daily dose difficult. In such cases, for example, a combination of hydrophilic polymers, one of which may be ionic in nature, is used to design ER matrices with a more prolonged drug release profile as compared to formulations where single polymers are used [49,92–97].

2.2.1 Compatibility and Miscibility of Polymers

The functional properties of the blends depend upon the miscibility of the polymers at the molecular level, i.e., preference is given to the blends with mutual attraction of macromolecules in dilute aqueous solutions compared to immiscible ones [85,87]. Miscibility will only occur when a strong specific interaction is present within the dilute solution system, such as random dipole, induced dipole, dipole-dipole, ion-dipole, H-bonding, acid-base, and charge transfer interactions. For example, XG and Na CMC have been shown to be incompatible due to the presence of the enzyme cellulase in the former, causing breakdown of Na CMC structure, resulting in a decrease of viscosity [98]. Whereas the combinations of ionic polymers (i.e., Na CMC or carbomer) with non-ionic hypromellose were found to produce synergistic interactions leading to an unexpected viscosity increase [99].

The mechanism of this type of interaction would normally depend on factors such as crosslinking of H-bonds between hydroxyl groups, nature of the polymer, degree of substitution, polymer chain configuration and length [98]. Generally, the crosslinking between different species would be greater compared to interactions between similar molecules due to formation of stronger hydrogen bonding. For instance, the H-bonding between a carboxyl group of Na CMC and a hydroxyl group of HPMC would usually be greater than between two hydroxyl groups of HPMC. The H-bonding would also be more prominent in the longer side-chain polymers, where a larger number of groups is available for the interaction to occur.

Thermodynamic interaction parameters can be calculated using the Flory-Huggins theory of mixing. In a study by Fuller *et al.* [100], looking at the interactions in PEO and HPMC blends, a Flory-Huggins interaction parameter, x_{12} (which was defined to describe the enthalpy of mixing), was related to the interaction energy density (B), the gas constant *(R)*, the observed equilibrium melting point of blended PEO (T_m) and to the molar volume of the repeating units of HPMC (V_{1u}); where subscripts 1 and 2 refer to HPMC and PEO, respectively (Equation 2.1). The HPMC-PEO films made from *N*,*N*-dimethylacetamide (DMAc) and water yielded negative values for the interaction parameter, indicating a miscible blend [100].

$$\mathrm{B} = RT_m\,(\chi_{12}/V_{1u}) \tag{2.1}$$

Karavas *et al.* [101] analyzed drug release from miscible polymer blends (PVP/HPMC and PVP/chitosan) prepared using the solvent evaporation technique. DSC studies revealed that both blends were miscible in the entire composition range because only one glass transition temperature was detected in polymer mixtures. Miscibility was attributed to the strong hydrogen bonding interactions between the carbonyl group of PVP (which acted as a strong proton acceptor) with hydroxyl and amino-groups of HPMC and chitosan (which were proton donors) verified by FT-IR. The authors reported that the extent of interactions not only depend on the type of reactive groups involved in the reaction, but was also based on the polymer's composition and ratio. These new polymer blend systems were able to produce matrices with completely different physical properties in order to use as effective drug carriers. For example, PVP/HPMC blends were produced as pulsatile chronotherapeutics systems, exactly adjusting the time of the drug release, whilst PVP/chitosan blends were produced to control the release profile of a poorly water-soluble drug.

2.2.2 Combination of Non-Ionic Polymers

Mixtures of different cellulose ethers have been widely employed in oral ER hydrophilic matrices [9], such as blending of different viscosity grades to achieve intermediate viscosity grades [90,102]. For example, the release of the practically water-insoluble drug nifedipine is significantly dependent on hydrodynamic conditions when HPMC (K100LV) alone is used, however, a combination of two HPMC grades (K15M and E15) increases the robustness of the matrices whilst still producing similar dissolution profiles at various agitation speeds during dissolution testing [90]. Hardy *et al.* [103] have also used blends of HPMC and PVP in a hydrophilic matrix to achieve a biphasic release of water-soluble caffeine. Feely and Davis [104] investigated the effect of PEG 6000 and EC on chlorpheniramine release from hypromellose matrices. The authors found PEG and EC to play different roles in the drug release process. PEG was found to have acted as a swellable polymer promoting water uptake into the tablets, whereas EC appeared to have behaved as an inert diluent having no significant effect on drug release.

According to Liu and Fassihi [105] and Gusler *et al.* [106], the use of PEO and HPMC in combination, provides beneficial performance in gastro-retentive drug delivery systems presented as triple- or bi-layer composite matrices. The swelling behavior of PEO in such formulations is balanced and better controlled by the HPMC, whilst the PEO

suppresses the faster erosion behavior of HPMC due to its rapid initial swelling, thereby minimizing/eliminating a potentially high initial drug release [105–107].

Fuller *et al.* [100] investigated blends of PEO and HPMC in the form of casting films for specific polymer-polymer interactions. According to the authors, such interactions may affect the drug release behavior of compressed matrices containing these polymers. The Flory-Huggins theory of mixing interaction parameters for HPMC:PEO blends was calculated and was found to be negative (-0.4 ± 0.1) for a water cast film. Negative value indicated that two polymers were miscible. In addition, FT-IR and Raman spectra confirmed hydrogen bonding interactions between the hydroxyl group of HPMC and the ether group of PEO. Other non-ionic polymer combinations include PEO and PEG with and without counter polymer (i.e., crosslinked carboxyvinyl polymer-Carbopol 971 P NF), proving PEO/PEG matrices as an effective way of achieving the ER of a large dose of highly water-soluble drugs and reducing the effect of pH on the release profiles [108]. Mahalingam *et al.* [109] also manipulated the swelling, bio-adhesion and rate of drug release from PEO gastro-retentive matrices by mixing it with non-ionic PVP. It was claimed that the gastro-retentive bahavior enabled the dosage form to reside in the stomach for more than one hour, and as a result, increased bioavailability of the drug (both water-soluble and water-insoluble candidate drugs).

2.3 Combination of Non-Ionic with Ionic Polymers

The combination of HPMC with anionic polymers such as Na CMC [49,86,110,111], sodium alginate [112–115], polymers of acrylic acid or carbomers [99,116,117]; enteric polymers, i.e., polyvinyl acetate phthalate [5,89,118], methacrylic acid copolymers [81,92,93,119] and natural gums like carrageenan, GG, locust bean gum (LBG) and XG [120–124], have been extensively studied, mainly for highly water-soluble drugs. Incorporation of cationic polymers into HPMC matrices has also been investigated, specifically using Eudragit E100 [5].

Khanum and Gayathri [125] evaluated ER matrix tablets of diclofenac sodium using blends of PEO and XG and found that appropriate proportions of the polymer mixtures could produce a more prolonged drug release compared to single polymers. Several other authors have investigated drug release from PEO in combination with Eudragit, HPC, sodium alginate, PEG, PVP, anionic carbomer (Carbopol) and HEC, either by direct compression or wet granulation methods, and found the polymer mixtures to successfully achieve ER patterns [87,125–131].

2.4 Combinations of Ionic Polymers

Cationic polymers such as dimethylaminoethyl methacrylate copolymer (Eudragit® E100) and butylated methacrylate copolymer (Eudragit® EPO) have been used with acidic drugs (i.e., divalproex sodium) and/or with anionic polymers (i.e., PVAP) for the development of pH-independent release [5,90,132,133].

The ER matrices containing a mixture of anionic XG and cationic LBG with cationic drugs such as metroprolol tartrate [134] or P-HCl (P-HCl) [135] have shown better

control of drug release in comparison to those tablets where single polymers were used. This has been related to the synergistic interaction between two polymers, allowing uniform tablet hydration and elimination of burst release. Asghar *et al.* [136] assessed the combination of cationic GG with pH sensitive anionic polymers (Eudragit L100 and S100) matrices for colonic delivery of acidic drug indomethacin. The inclusion of Eudragit into GG matrix base significantly reduced the drug release in the initial phase (0–6 h), followed by controlled drug release up to 14–16 hours. This sigmoidal (biphasic) release pattern observed from the designed formulation was the most suitable for colonic delivery and had clear advantages over the performance of single polymer formulations. The effect of combining three polymers, carbomer, PVAP and HPMC, on guaifenesin release was reported by Tiwari and Rajabi-Siahboomi [90]. Tablets containing 15% of the polymer blend produced drug release similar to formulations containing 30% HPMC alone. The results indicated similar release profiles to formulations with single polymer using polymer combinations at overall lower polymer inclusions. This may lead to the improved processing, allow for accommodation of a higher dose/use lower tablet weight together with reduction of overall cost of the product. Using a similar approach, Siahi-Shadbad *et al.* [137] formulated P-HCl in a tertiary polymer mixture (HPMC, psyllium and sodium alginate) and found that varying the concentration of each individual polymer had the ability to manipulate the release profile as well as having a better control of the burst release.

2.5 Other Polymer Combinations

There are studies that show that the inclusion of anionic surfactants, hydrophobic polymers, waxes, fatty acids, alcohols and ion exchange resins in HPMC ER matrix formulations affect drug release due to possible drug-ionic excipient interactions [90,104,138–141]. Combinations based on a directly compressed matrix consisting of drug and hydrophobic triacetyl-ß-cyclodextrin (TAßCD) dispersed in a polymeric material (HPMC, XG, chitosan, EC, Eudragit L100-55) was successfully employed in order to increase absorption of drugs with low bioavailability and short half-life, such as metformin HCl, in the upper gastrointestinal tract [142]. Other authors have modulated the drug release of metroprolol succinate or P-HCl by inclusion of an insoluble polymer (EC) into a hydrophilic matrix (HPMC) in order to extend the drug release using different polymer combinations [143,144]. According to Gallardo *et al.* [145], by combining differently charged (meth)acrylate copolymers, different release patterns can be achieved due to polymer interaction resulting in new polymer properties and providing further advantages in the processing and/or modulation of the release profiles.

2.6 Effect of Dissolution Method (Media) on Drug Release from ER Matrices Containing Polymer Combinations

Takka *et al.* [92] reported that the dissolution of P-HCl from HPMC:Na CMC matrices may be complex. The blends of 3:1 and 1:1 HPMC:Na CMC gave a slower drug

release compared to the formulation that contains only HPMC. The authors suggested that one of the reasons for this phenomenon was due to potential increases in the viscosity of the gel layer by addition of Na CMC to a non-ionic cellulose like HPMC, through strong hydrogen bonding between the carboxyl groups of Na CMC and the hydroxyl groups of HPMC, leading to strong crosslinking between the two polymers [92], and as a result, lower drug diffusion (release rate) from the matrix. The authors also postulated that a decrease in drug release was probably related to the formation of a complex between the cationic drug (P-HCl) and the anionic Na CMC. Similar results were also reported by Dabbagh *et al.* [49] and Palmer *et al.* [94,95]. Matrices containing pure Na CMC showed a fast release of P-HCl because of tablet disintegration, i.e., more than 80% of the drug in the matrices was released during the initial five minutes of contact with dissolution media. The rapid erosion of the matrices may be due to the higher solubility of NaCMC compared to HPMC, and high aqueous solubility of the active. The same authors showed that drug release rate (% $h^{-1/2}$) values for the formulations containing binary mixtures of polymers were significantly lower (approximately by up to 2-fold) in comparison to values from matrices containing only one polymer in the formulation (Figure 2.1). A much more significant synergy (more prolonged drug release from binary formulations) was observed in water compared to phosphate buffer pH 6.8, and no unexpected results were produced in acid. At low pH (3.0 or less), less than 10% of the Na CMC acid groups will be ionized, resulting in relatively little stiffening by electrostatic charge repulsion, and relatively little swelling compared to fully neutralized NaCMC. Possibly, as the pKa of Na CMC is 4.3, then above that point, the carboxylic acid groups are ionized to a great extent, thus facilitating interactions with the protonated NH group of the drug (P-HCl) or the OH of PEO. In addition, the carboxylate groups on the polymer backbone ionize, resulting in repulsion between the anions and further increasing the swelling of the polymer and thus the reduced release rates. The latter case could contribute to the slower release profiles [146].

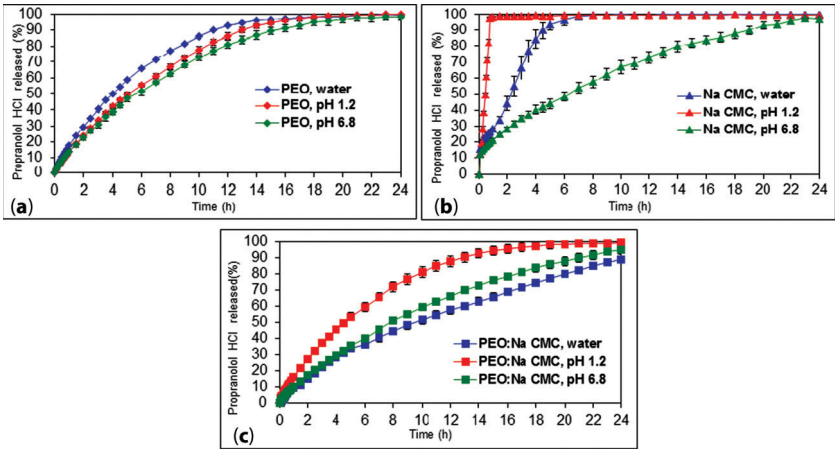

Figure 2.1 P-HCl release from PEO (Coagulant) and NaCMC (HP-5HS) matrices in various dissolution media (100 rpm, USP II, sinkers, 900 mL water) [147].

2.7 Main Mechanisms of Drug-Polymer and/or Polymer-Polymer Interaction in ER Formulations

Oppositely-charged molecules attract, while similarly-charged molecules tend to repel each other [148]. Binding interactions can be physical or chemical. Physical bonding occurs due to electrostatic interactions, hydrogen bonding or van der Waals forces and is usually reversible; while in chemical adsorption, the adsorbate is attached to the adsorbent by primary chemical bonds, including ion exchange, protonation and complexation, and it is irreversible. Several adsorption mechanisms can operate simultaneously, depending on the material nature and concentration and/or media pH, ionic strength and temperature [149]. For example, the sorption of acidic drugs, such as salicylic acid, by the Eudragit RL and RS polymers is primarily due to ionic electrostatic interactions. The exclusive adsorption of anionic drug molecules to the Eudragit RL polymer indicated similar functionality to an anion exchange resin containing quaternary ammonium groups [150]. Bound drug could be liberated by decreasing the pH or increasing the ionic strength of the media to decrease the electrostatic binding interactions between the anionic drug and polymer (Figure 2.2). Because both reversible and irreversible binding of salicylic acid were observed during desorption studies, authors suggested that there is a presence of more than one type of binding interaction. The van der Waals forces and solubilization of drug by the polymer have been described as secondary possible mechanisms of interaction [6].

The interaction between the drug (*API*) and anionic polymers (*AP*) is an equilibrium reaction and 1:1 complexation (*API-AP*) is assumed; Equation 2.2 may be presented as [92]:

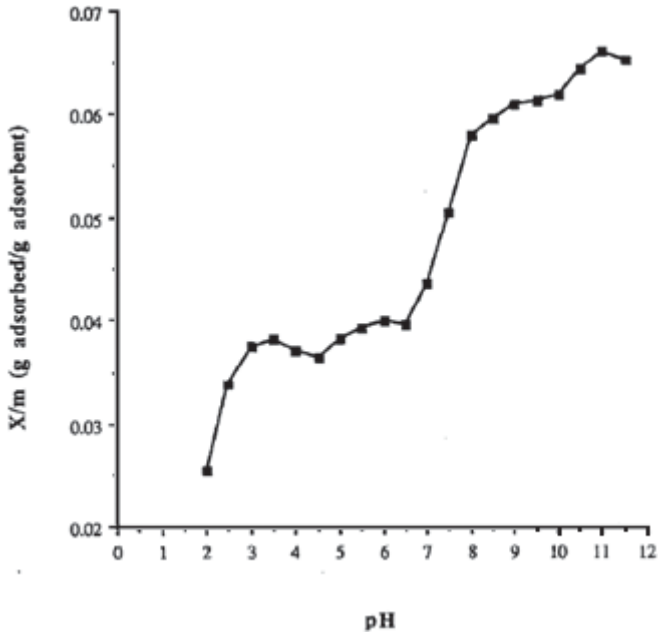

Figure 2.2 The influence of pH on the sorption of salicylic acid from a 1 mg/ml solution to Eudragit RLl00 in 50 mM sodium phosphate buffers. Adapted with permission from [149].

$$API + AP \overset{K}{\longleftrightarrow} API - AP \tag{2.2}$$

The system response (UV absorbance and concentration) of complex, drug and anionic polymer can be measured (i.e., using equilibrium dialysis or UV method). That response is then related to the relative concentrations of free and bound drug and subsequently to the drug polymer binding constant (K), as defined in Equation 2.3 [151].

$$K = \frac{Complex}{DrugxAnionicPolymer} \tag{2.3}$$

Takka *et al.* [92] carried out UV spectroscopy experiments of various drug-ionic polymer aqueous solutions at controlled pH of 6.8. All samples were scanned and the absorbance spectrum of an unbound drug was measured with the binding constant, K, to be 3785, 3819, and 6274 for P-HCl-Eudragit S, -Na CMC, and -Eudragit L100-55 complexation, respectively (Figure 2.3). The authors postulated that P-HCl-anionic polymer interaction was depended on the COOH groups of the anionic polymers. The ascending rank order of the binding constant of P-HCl to anionic polymers was as follows: Eudragit S < Na CMC < Eudragit L100-55. The ratio of the free carboxyl groups to the ester groups was also an important parameter for the complex formation, which was approximately 1:1 in Eudragit L 100–55 and about 1:2 in Eudragit S. Dittgen *et al.* [152] reported that the ion exchange capacity of Eudragit L 100-55 was around 6 mEquiv/g of polymer and 3.5 mEquiv/g in the case of Eudragit S, which was similar to the Takka *et al.* [92] results. According to Takka *et al.* [92], the lower binding constant of Na CMC compared to Eudragit L 100-55 could be due to the larger molecular weight of the Na CMC; as such the interaction between P-HCl and Na CMC was more complex than with the methacrylic acid copolymers. Tucker *et al.* [153] found that

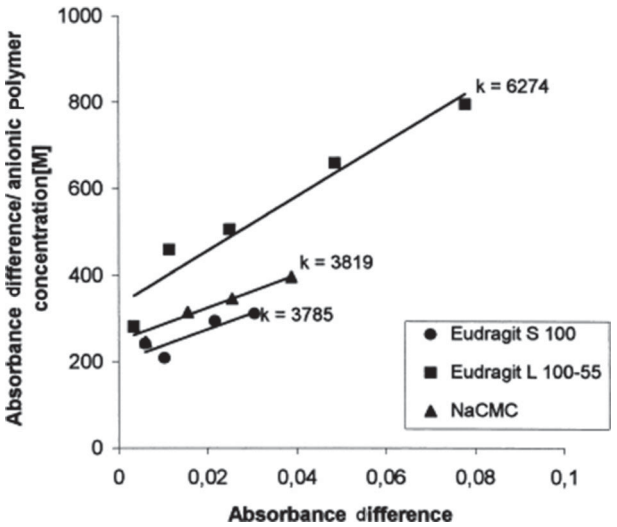

Figure 2.3 P-HCl-anionic polymer complexation. Adapted with permission from [92].

one propranolol cation would be bound to each carboxyl anion of Na CMC. Dabbagh *et al.* [49] reported that each sodium ion in Na CMC can potentially be replaced by a propranolol moiety, but the number of sites of sodium that require replacement before precipitation is unknown. Therefore, it is difficult to create a model to describe the binding interaction between P-HCl and Na CMC, and in order to confirm the existence of this interaction between cationic drug and anionic polymers, investigations have to be pursued by using various physicochemical methods.

Similar results were obtained by Palmer [147] for PEO:Na CMC and PEO:XG P-HCl matrices using equilibrium dialysis. This is a technique that is used to detect material interactions and degree of binding between small molecules or ions and biopolymers, involving separation of the unbound ligand. The method was first reported by Davis [154] and Klotz [155], who studied the binding of low molecular weight compounds to biopolymers. It works on the principle that a solution of high molecular weight compound is separated from a solution of low molecular weight compound (drug) by a semipermeable membrane, selected to allow free diffusion of the drug, but retention of large polymer molecules [148]. As the Na CMC or XG content in the dialysis bag increased, the free drug in the fluid outside the bag decreased, and subsequently the percentage of P-HCl bound to the anionic polymers increased. The binding constant (K) was determined by linear regression. It was found that two grades of Na CMC had significantly higher (2.6 times) binding constant values (K = 6.52, 6.61) compared to xantham gum (K = 2.54, 2.60) in water. No interaction was observed in acid (pH 1.2) and buffer (pH 6.8), possibly due to non-ionization of drug or/and polymer in these conditions. The results indicated that polymer nature was the main factor in drug-polymer binding interaction. Drug release from these matrix systems is also dependent on the nature of the active substance used [94,95]. For the anionic ibuprofen and the non-ionic theophylline, no unusual/unexpected release profiles were obtained from matrices containing a mixture of PEO and Na CMC (Figure 2.4). However, for the cationic salt P-HCl, a combination of two polymers produced a significantly slower drug release (beyond 12 h), compared to the matrices with single polymers. Palmer *et al.* [95] also investigated the release of chlorpheniramine maleate, venlafaxine HCl and verapamil HCl (V-HCl) from PEO and Na CMC matrices. The authors found relatively faster drug release occurring from matrices containing only Na CMC (100% in 6–8 h). PEO matrices had a significant slower release (100% in 13–18 h), with the matrices containing mixtures of PEO and Na CMC having the lowest release (100% in 16–30 h) compared to the formulations based on single polymers. It was postulated that there may be interactions between the drug and Na CMC, resulting in drug-polymer with a lower aqueous solubility.

The DSC thermograms of the P-HCl complexes in the presence or absence of PEO, physical mixtures and the bulk substances (Figure 2.5) were analyzed. The DSC heating curves of pure NaCMC and PEO exhibited a glass transition temperature at 121.70°C (ΔH = 265.90 J/g) and a melting point at 77.09°C (ΔH = 214.26 J/g), respectively. The pure API showed a single endotherm corresponding to the melting of the drug at 169.67°C (ΔH = 121.96 J/g). No interaction between cationic P-HCl and anionic Na CMC was observed in dry powder form (with or without presence of PEO). However, the P-HCl:Na CMC complex in the presence of PEO produced a broad melting endotherm of P-HCl at 213.80°C. In addition, the Tg of NaCMC was lowered at 105.85°C, while the melting peak of PEO disappeared or merged with the Na CMC peak.

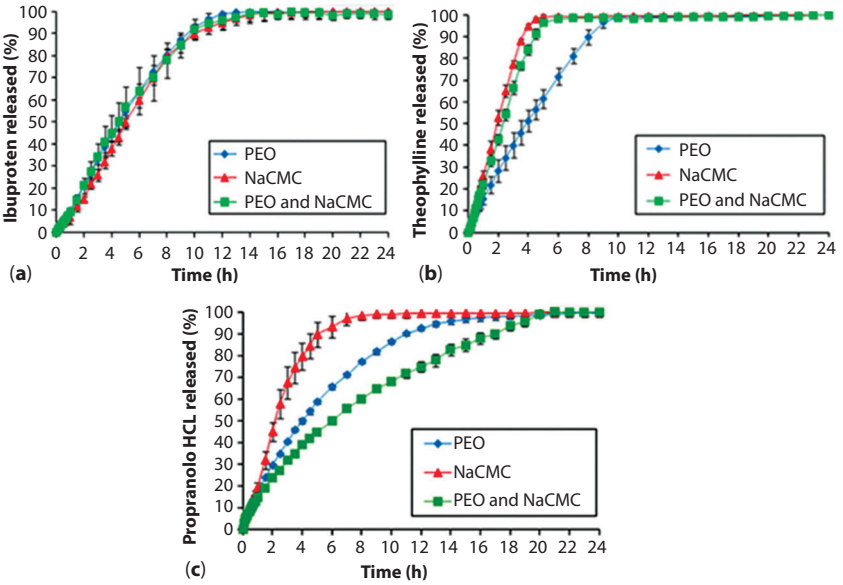

Figure 2.4 Effect of Na CMC on (a) ibuprofen in pH 7.2 phosphate buffer and (b) theophylline, and (c) P-HCl release from PEO ER matrices in water. Adapted with permission from [94].

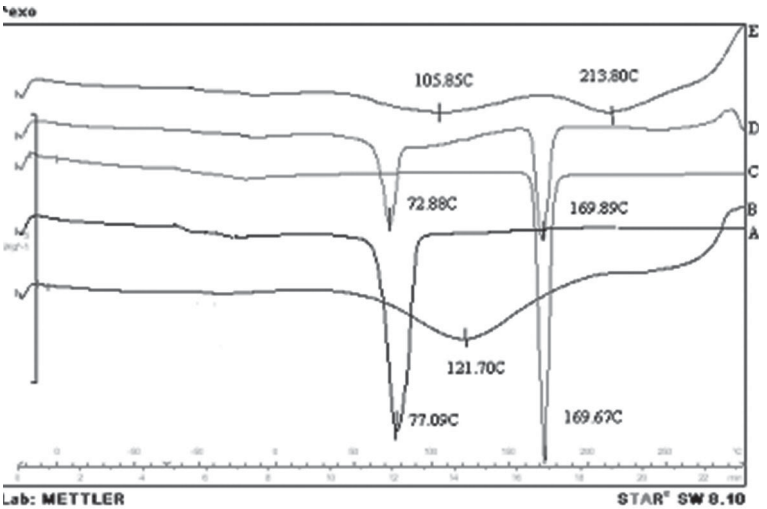

Figure 2.5 DSC heating curves for dry powders: (A) PEO, (B) NaCMC, (C) P-HCl, (D) P-HCl, PEO and Na CMC; and (E) P-HCl, PEO and Na CMC complex. Adapted with permission from [95].

Complex formation between antidiabetic drugs (gliclazide and repaglinide) and human serum (albumin) and/or cyclodextrines with proteins is a reversible dynamic process (the rates for formation and dissociation within the drug complexes are continuously being formed and dissociated) without change in biological activity of the drug [156–159]. The same trend was seen by Palmer *et al.* [94] (Figure 2.4c), where *in-vitro*

data showed that the amount of drug released from formulations containing polymer combinations was about 98–100% beyond 24 hours, which indicated the reversible nature of the complex-formation and no change in the chemical nature of the drug.

The Na CMC, although neutral in nature, can dissociate into Na^+ ion and CMC^- ion, becoming negatively charged and so-called anionic polymer. P-HCl exists in a non-ionic state as a salt, however, it has an ability to lose and exchange its chloride atom and attract carboxylate anion from CMC under suitable conditions and form a complex [92,93]. An intermolecular interaction between the amine group of the cationic propranolol with the carboxyl group of the anionic polymer CMC, produced a less water-soluble salt, i.e., propranolol H^+CMC^-. This leads to a more prolonged drug release as compared to the non-complexed drug from matrices without Na CMC.

Table 2.1 shows the appearance of new peaks in complex formations with characteristic bands of carboxylic acid group vibrations at 1,488.81 cm^{-1} (possible –C=C– and –C=O vibration/stretch and –NH deformation) and 1,829.00 cm^{-1} (carbonyl COOH

Table 2.1 FT-IR spectra for dry physical mixtures (powder) and complex of P-HCl and NaCMC (for more description refer to [194]).

P-HCl /Na CMC (Powder)	P-HCl /NaCMC (Complex)	Comments
	689.59	New peak
	706.98	New peak
736.68	733.56	
769.46	770.19	
796.76	793.87	
900.25	900.09	
	990.3	New peak
1,029.92	1,026.53	
	1,038.15	New peak
1,105.06	1,101.25	Band width change
1,141.66		
1,156.11		Change in S = O symmetric stretching
1,240.46	1,240.17	
1,266.67	1,268.03	Band width change, C–O Stretch
1,321.89	1,314.39	Band width change, C–O Stretch

(*Continued*)

Table 2.1 Cont.

P-HCl /Na CMC (Powder)	P-HCl /NaCMC (Complex)	Comments
1,400.15	1,392.10	Band width change
1,452.14	1,456.80	Band width change
	1,488.81	New peak: –C = C– and –C = O vibration/ stretch and –NH deformation
1,509.64	1,507.65	
1,521.15	1,521.14	
1,580.14	1,580.13	
1,845.13	1,829.99	Carbonyl COOH stretching, C = O stretch; salt formation
1,918.79	1,918.53	
	1,923.96	New peak
1,943.99	1,943.59	
1,968.54	1,969.12	
	2,134.36	New peak
2,177.05	2,164.05	Stretch
	2,347.08	New peak
	2,732.59	New peak
	2,748.55	New peak
	2,850.38	New peak, –C–H
	3,249.04	New peak
3,271.55		
	3,330.58	New peak, –NH and –OH stretching

stretching, C=O stretching), which may be the result of salt formation. The absorption bands characterizing intermolecular bonding in the complex due to –C–H and –OH or –NH were observed at 2,850.38 and 3,330.58 cm^{-1}, respectively [94]. Similar results were reported by Takka for P-HCl matrices containing Eudragit (S100 and L100-55) and Na CMC [93]. The author claimed that when ionization occurred, the resonance between the two C–O bands together with formation of the COO– groups was possible, and stated that conversion of carboxylic acid into a salt was carried out by the

addition of an amine group to the former solution, confirmed by DSC and FT-IR analysis. Similarly, Sriwongjanya and Bodmeier [138] claimed that for the P-HCl and anionic exchange resin (Amberlite® IRP 69) in HPMC (various grades of METHOCEL) tablets, retarded release was mainly due to a drug-resin complex formation *in situ* within the matrix gel regions.

In another study, Elkheshen [146] showed that the drug-polymer interaction between the tertiary amine nitrogen of V-HCl with the anionic carboxyl group of Carbopol 934P formed an insoluble complex leading to a reduced rate of drug release. Differential scanning calorimetry (DSC) spectra showed that water-insoluble precipitate resulting from the interaction of Carbopol 934P and V-HCl had a melting point higher than 300°C, whilst the melting point of V-HCl was 143.77°C. The disappearance of the endothermic peak of the drug at 143.77°C from the thermogram of the interaction product, but not from that of the physical mixture, confirmed drug-polymer interaction to form the complex. The Fourier transform infrared spectroscopy (FT-IR) spectrum of the same materials supported the DSC results. The drug showed a strong band at 2850–2250 cm^{-1} due to the $N^{+}H$ stretch of the tertiary amine hydrochloride salt. The same band existed in the physical mixture, but only appeared as a weak band in the complex [146].

The disappearance of the strong band at 2850–2250 cm^{-1} suggested the absence of the drug hydrochloride salt in the product, while the appearance of the weak band suggested the formation of a salt between the high molecular weight acidic polymer and the relatively small molecules of the drug according to the scheme presented in Figure 2.6.

In addition, drug-polymer interaction led to neutralization of the carboxyl groups of the ionic polymer and suppression of the electrostatic repulsion between its anionic groups, which reduced the uncoiling of the polymer and chain relaxation, which lead to a decreased matrix swelling [146].

The decrease in the P-HCl release rate from PEO and Na CMC matrices could also be related to polymer-polymer interaction. According to Çaykara and Demirci [85], blending of non-ionic polyvinyl alcohol and anionic sodium alginate prepared by the solution casting method resulted in a polymer-polymer intermolecular interaction, the strength of which would be dependent on the compatibility or miscibility between the two mixed materials at a molecular level. Elmowafy *et al.* [160] analyzed blends of polysaccharides (κ-carrageenan and gellan gum) and cellulose ethers (hydroxypropylmethyl cellulose, hydroxypropylcellulose, sodium carboxymethyl cellulose) in an attempt to optimize matrix integrity and modulate the release characteristics of famotidine by formulating a single-unit floating matrix by a direct compression technique. The DSC and FT-IR studies revealed that changing the polymer matrix system by the use of polymer blends

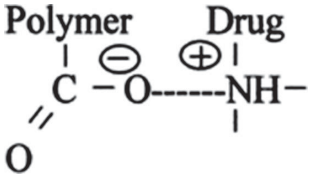

Figure 2.6 Schematic representation of polymer-drug interaction.

resulted in the formation of molecular interactions which may affect drug release. For example, authors reported H-bonding between κ-carrageenan and different cellulose ethers; formation of ionic bonding between the carboxyl groups of gellan gum and Na CMC in addition to a partial covalent bonding between oxygen atoms of ether groups and sodium ions. They also claimed H-bond formation between NH_2 groups of famotidine and OH groups of gellan gum together with ionic and H-bonding between drug and carrageenan, which lead to the retardation in drug release.

Sakeer *et al.* [161] investigated the use of XG and its binary blends with carbopol in order to obtain ER formulations of buccoadhesive nystatine tablets. The release rates of nystatin from tablets containing binary mixtures of both XG with any one of the tested carbopol grades were slower compared to the release behavior of the formulations containing only one polymer. Binary mixtures of carbopol-XG did not demonstrate 'burst' release and the presence of XG also appeared to maintain the integrity of the tablets during dissolution test. This allowed the carbopol to hydrate slowly in a more controlled manner, thereby promoting a slower release of the drug compared to matrices where carbopol was used alone as a matrix former. A comparison of the FT-IR spectra of pure nystatin, carbopol 934P and XG with the spectra obtained for the physical mixture of the two suggested interaction between nystatin and the polymers due to hydrogen bonding involving C=O, C-C-O and O-C-C chemical groups [161]. Sakeer *et al.* [161] also reported that swelling of buccoadhesive nystatin tablets depended on the nature, ratio and total concentration of diluents used in the formulation. For example, the formulation containing 60% of microcrystalline cellulose (MCC) showed the highest degree of swelling, followed by formulation containing 30% MCC and 30% lactose, whereas the minimum degree of swelling after 3 hours was displayed by the formulation containing 60% calcium carbonate. According to authors, the presence of dissolved calcium carbonate in matrices increased the pH around XG, and as a result, increased the ionization of carboxylic groups on the trisaccharide side chains of XG. It has been reported that Ca^{2+} ions can be condensed around the ionized carboxylic moieties of XG, reducing the repulsive forces between polyelectrolyte chains and leading to lower intermolecular repulsion, as well as changing water arrangement [162]. The trisaccharide side chains of XG in the presence of calcium ions probably collapsed down to the backbone, resulting in the change of the polymer structure, i.e., the XG molecule becomes a rigid rod-like shape [163]. In addition, complexation between the divalent Ca^{2+} and the polyionic XG side chains leads to a decreased flexibility of the polymer chains by partially holding them together by calcium ions, promoting a more rigid structure, and as a result, any interpolymer hydrogen bonding between XG polymer chains is also reduced [164,161]. All these changes resulted in a reduced ability of XG molecules to interact with water; therefore swelling was reduced for the XG matrices containing calcium carbonate [161].

To further understand the mechanisms of complexations, Palmer *et al.* [95] went on to use a non-destructive spectroscopic technique, X-ray photoelectron spectroscopy (XPS), to investigate the interactions of the drug (P-HCl)-polymer (Na CMC) complex. This technique provides a quantitative determination of the elemental surface composition of the tablet matrix. The peaks' fits of C (1s), O (1s), N (1s) and Cl (2p) are shown in Figure 2.7. For the analyzed API:Na CMC:PEO hydrated tablet, C (1s) peak at 288.48 eV is a strong indication of an amine group attached to the carbon atom,

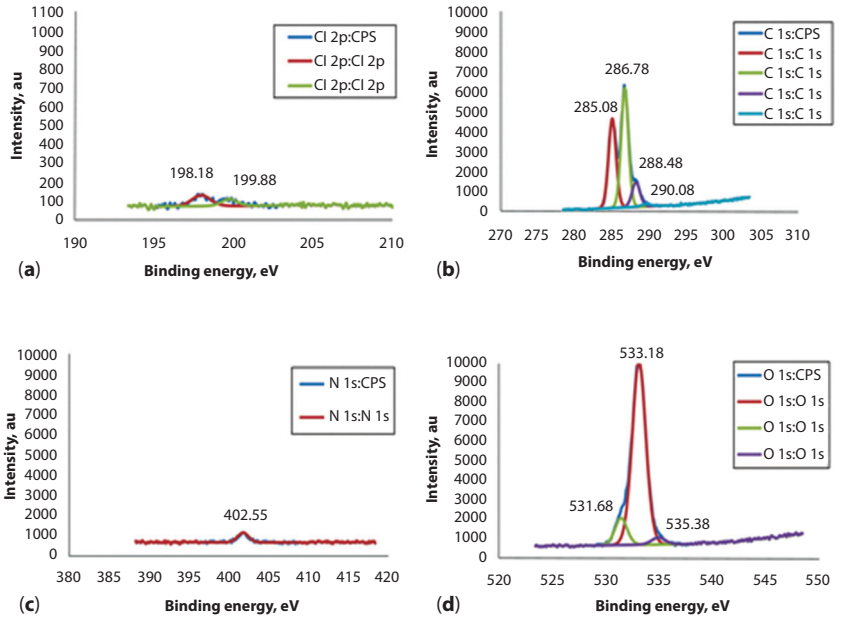

Figure 2.7 XPS spectra for the (a) Cl (2p), (b) C (1s), (c) N (1s) and (d) O (1s) binding energy peaks (eV) for complex formation of Na CMC with P-HCl. Adapted with permission from [95].

whilst the N (1s) peak at BE = 402.55 eV suggests additional protonation of a secondary amine group (NR_2H^+) of the drug previously reported by Beamson and Briggs [165], and indicating the formation of hydrogen bonding [95]. The absence of additional N (1s) binding energy peaks indicates the absence of ionic or covalent linkages [166]. Furthermore, the BE of Cl^- atom is shifted at BE = ~200 eV representing Cl^-H^+ interactions (very poor signal). An interesting change was observed at BE = 535.18 eV and BE = 535.38 eV for O (1s) atom, where O is attached to a Na moiety with significant peak shifts. Therefore, the authors proposed that the primary mechanism of interactions detected by XPS between amine group of API (P-HCl) and ester/carboxyl group of polymer (Na CMC) was due to the formation of protonated amine and its linkage with the carboxyl/ester shifted O atoms through the proton attached to N atom in active ammonium through hydrogen bonding [95].

On the other hand, hydrated PEO or Na CMC tablets (Figure 2.8) [147] showed two peaks at BE = ~286.59 eV and ~285.19 eV, which predominantly represented –C-COOH/H-O-C-CH and C-C-COOH/-C-C structures, respectively. Similar peaks were produced for Na CMC hydrated tablet, with an additional broad peak of a low intensity at BE of ~288.61 eV, possibly due to the –COOH/COO- structures. Interestingly, hydrated tablet containing both PEO and Na CMC (without API) showed a peak shift at BE of ~287.31 eV, where all C-C bonds were converted to more stable –COO/-COO-COH structures. This value of the peak shift at ~287.31 eV was about ~0.72 eV, strongly supporting the formations of C-O-O- structures.

Palmer *et al.* [95] analyzed the potential additional polymer-polymer interactions, which may be due to the presence of the PEO potentially interacting with NaCMC, by

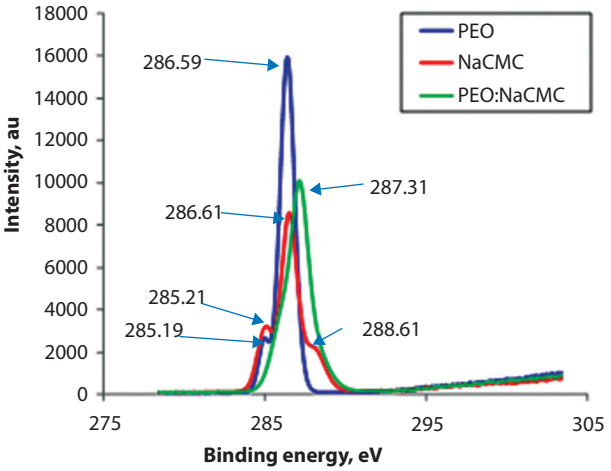

Figure 2.8 XPS spectra for the hydrated tablets of PEO (Coagulant, Dow, USA), NaCMC (HP-12HS, DKS, Japan) and PEO:Na CMC (without P-HCl) [147].

analyzing the binding enthalpy and the association constant values using high sensitivity isothermal titration calorimeter (ITC) by injecting drug (P-HCl) into polymer (Na CMC or PEO) or polymer (PEO) into polymer (Na CMC). The ITC studies confirmed the strong bonding between NaCMC and propanolol HCl ($K = 2.95 \times$ E11 M^{-1}) but also revealed interactions between NaCMC and PEO ($K = 4.84 \times$ E12 M^{-1}) which have not been reported elsewhere.

Various values (ΔH, ΔS and ΔG) were depicted from the ITC studies. ΔG is directly related to the binding affinity, and when ΔG values are negative, suggests spontaneous interactions, whilst more negative ΔG values indicate stronger interactions. In addition, ΔH reflects the strength of the drug-polymer or polymer-polymer interaction relative to those with solvent, primarily due to hydrogen bond formation. When ΔH is negative, the interactions are enthalpically favored, while positive $T\Delta S$ values result in entropically favored interactions. All drug-polymer and polymer-polymer interactions were found to be negative (ΔH = −1.71 E11 and −1.41 E12 for the combinations of Na CMC with P-HCl or PEO, respectively), hence spontaneous hydrogen bond formation. The Na CMC-PEO high binding constant provided further insights about the interactions that take place when the tablet formulations are hydrated. The reduced drug release rates can then be attributed to the additional polymer-polymer interactions that reduce the drug molecule mobility through a newly formed network. No interaction was observed during the titration of P-HCl with non-ionic PEO [95].

2.8 Summary and Conclusions

Polymer combinations with positively charged active substances can produce significantly slower release compared to the matrices where single polymers are used. This can primarily be attributed to hydrogen bonding, leading to a reversible drug-polymer

complexation with a formation of a new form/type of active (salt), having more prolonged release compared to the original active substance.

This synergy between materials can be used to control the release patterns of freely and/or very water-soluble drugs, especially when administration of a high dose is required and/or once daily dose is preferred. An additional synergistic polymer-polymer interaction can enhance drug-polymer bonding and produce more prolonged and/or near zero-order release rates from polymer blends. These findings indicate the appealing nature of the use of simple binary mixtures of polymers to industry and are as such being used more and more to tackle the issues associated with the use of high single polymer content in slow release tablet matrices. Furthermore, the decrease in initial burst release and obtaining near zero-order release with the use of binary mixtures of polymers is an additional benefit which has attracted the attention of researchers in the field for the further development of slow release matrices.

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3

Reagents for the Covalent Attachment of mPEG to Peptides and Proteins

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Abstract

In the last forty years, the conjugation of methoxy-poly(ethylene glycol) (mPEG) has evolved to become a well-established technology for the improvement of the physicochemical and therapeutic properties of biopharmaceutical peptides and proteins (PEGylation). In fact, eleven PEGylated products are available nowadays in the marketplace, while several others are under clinical evaluation.

Ubiquitously present nucleophillic groups, such as the terminal -NH_2 group, the ε-NH_2 group of lysine and the -SH group of cysteine, have all been used to couple peptides and proteins to mPEG derivatives. Moreover, site-selective, reversible and enzymatic PEGylation have recently gained increasing attention among the biopharmaceutical community. The aim of this chapter is to summarize the most relevant and recent achievements obtained in the PEGylation of peptides and proteins, with an emphasis on the chemistry underlying the currently available methods for the preparation of mPEG reagents, as well as the chemistry involved in the PEGylation reactions.

Keywords: PEGylation, bioconjugation, PEGylation chemistry, reactive PEGs, PEG-protein, protein PEGylation, PEG reagents

3.1 Introduction

Chemical conjugation with poly(ethylene glycol) (PEG) has evolved to become a well-established technology used to improve the physicochemical, biomedical and pharmacological properties of several therapeutic molecules, such as peptides, proteins, antibodies, antibody fragments, oligonucleotides, and small drugs [1]. On the other hand, PEG is a Food and Drug Administration (FDA) approved diol compound that has historically been used as excipient in pharmaceutical and cosmetic formulations and as a solvent in organic synthesis. However, PEGylation with PEG diol derivatives is usually limited to the conjugation of small molecules, which require increased drug loads per polymer chain. In fact, other PEG derivatives such as dendrimers and

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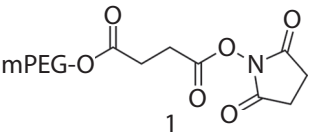
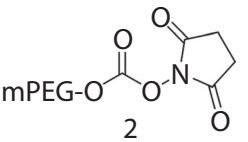
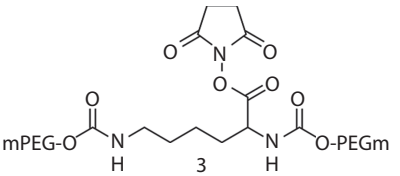
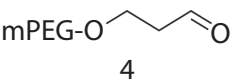
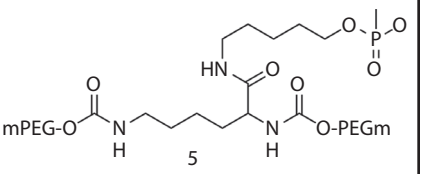
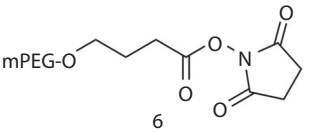
dendromers are preferred for small drug delivery purposes, owing to the fact that higher drug ratios can be achieved. In the PEGylation of peptides and proteins, the utility of PEG diol reagents for conjugation is relatively limited, since they can lead to the unwanted crosslinking of the conjugates, affording aggregates with increased inmmunogenicity. Methoxy-poly(ethylene glycol) (mPEG) is a protected version of PEG, which is more suitable for bioconjugation of peptides and proteins, since these monofunctional reactive polymers do not lead to the crosslinking of the poly-peptidic targets. mPEG is a biocompatible and non-biodegradable polymer whose properties make it well-suited for peptide and protein conjugation. In particular, the distinctive solubility behavior (i.e., mPEG is soluble in water, methylene chloride, chloroform, toluene, acetonitrile and other solvents; while it is not soluble in ethyl ether, *iso*-propanol and aliphatic hydrocarbon solvents) allows for the purification of mPEG and mPEG derivatives from low molecular weight organic impurities via a simple precipitation-centrifugation work-up. Although it is usually claimed that mPEG is not antigenic, immunogenic or toxic [2,3], the occurrence of antibodies against PEG (anti-PEG) has been reported when high polymer loads were evaluated [4,5]. The mPEG chains are flexible, and in aqueous solution they coordinate 2-3 water molecules per oxyethylene unit, meaning that mPEGs have apparent molecular weights that are 5 to 10 times higher than that of globular proteins of comparable molecular weights. Even though it has traditionally been considered that the polydispersity of mPEG would be a critical issue to obtain homogeneous conjugates, it is actually possible to use mPEG polymers with narrow polydispersities, which are available from different suppliers. Good quality mPEGs, with PDIs ranging from 1.01 for low molecular weight mPEGs (< 5000 Da) to 1.1 for mPEGs of up to 50 kDa, can now easily be acquired, and these polymers are well-suited for pharmacological applications (PDI: a measure of distibution of molecular masses, defined as weight average molecular weight (M_w) divided by number average molecular weight (M_n)).

Conjugation of mPEG to bioactive polypeptides and proteins increases plasmatic half-life of the biomacromolecules by increasing the hydrodynamic ratio, which in turn reduces excretion rate. Furthermore, it is widely-accepted that PEGylation decreases the immunogenicity by shielding antigenic epitopes, as well as reducing the degradation by proteolytic enzymes and antibody recognition by covering the recognition sites [6]. In addition, PEGylation increases water solubility, an effect that has been exploited mainly in the conjugation of low molecular weight drugs [7,8]. Conjugation with mPEG can also modify the biodistribution; promoting the accumulation of the conjugated biodrugs into tumors [9,10], and the accumulation in specific organs, tissues or cells by the enhanced permeability and retention (EPR) effect [11,12].

The -OH group of mPEG is not reactive enough to allow direct coupling of the polymer to biomacromolecules. Thus, it is necessary for this -OH group to be converted into a more reactive species that can react with a functional group that is present in the peptide or protein [13]. The ε-NH_2 of lysine, the terminal -NH_2 group, as well as the secondary -NH group of histidine and the -SH group of cysteine are all nucleophilic sites that have widely been employed for the reaction with electrophilic mPEG derivatives.

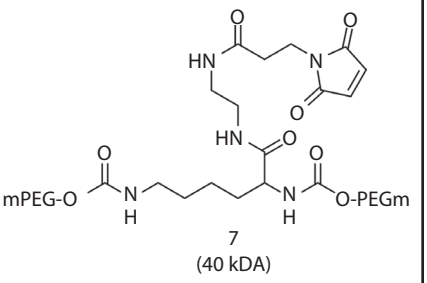
Since approval of PEGylated adenosine deaminase by the FDA, 10 PEGylated drugs have reached the market (Table 3.1), and most of these valuable conjugates are obtained using a few well-known electrophilic mPEG reagents [14].

Table 3.1 Commercially available PEGylated conjugates.

Year	Protein	Commercial Name	mPEG Reagent
1990	Adenosine deaminase	Adagen®	 1 (5 kDA)
1994	Asparaginase	Oncaspar®	1 (5kDA)
2000	Interferon alpha-2a	PEGintron®	 2 (12 kDA)
2001	Interferon alpha-2b	PEGasys®	 3 (40 kDA)
2002	G-CSF	Neulasta®	 4 (20 kDA)
2003	Growth hormone receptor antagonist	Somavert®	1 (5kDA)
2004	Oligonucleotide	Macugen®	 5 (40 kDA)
2007	Epoetin beta	Mircera®	 6 (30 kDA)

(*Continued*)

Table 3.1 (Cond.)

Year	Protein	Commercial Name	mPEG Reagent
2008	Anti TNF-Fab´	Cimzia®	 7 (40 kDA)
2010	Porcine-like uricase	Krystexxa®	2 (10 kDA)
2012	Synthetic peptide	Omontys®	3 (40 kDA)

Proteins are considered to be sensitive materials whose bioactivity can easily be altered, even under relatively mild conditions. Therefore, mPEG reagents should react with macromolecules under these mild aqueous conditions, within a relatively narrow *p*H range. Furthermore, it is necessary for the conjugate and the unreacted protein to be purified from the unreacted mPEG derivatives, the poly-PEGylated adducts and other reaction byproducts under mild and strictly controlled purification conditions. The purification of PEGylated proteins and peptides can be quite demanding and each purification step should be carefully defined, studied and optimized.

3.2 General Considerations about PEG Reagents and PEGylation Reactions

Nowadays mPEG is a readily available starting material that can be acquired from several different providers in good quality. Diol content and polydispersity index (PDI) are the two main factors that determine the quality of a given mPEG raw material. PEG-diol, which arises from trace amounts of water, oxygen or other impurities present during polymerization, can afford bifunctional reactive polymers, which can ultimately lead to the formation of undesired crosslinked conjugates [15]. The PDI is another critical issue that determines the quality of an mPEG raw material or an mPEG reagent. In general, PDIs as close as possible to 1.00 are desired, since more homogeneous and defined conjugates can be obtained, which can be characterized more easily. It should also be noted that high PDIs on the mPEG starting material (or mPEG reagent) may ultimately result in too polydisperse conjugates that can hinder the purification processes and affect the biological properties of the conjugates.

As indicated earlier, the low reactivity of the free -OH group of mPEG precludes its direct use in bioconjugation. Thus, the -OH group has to be transformed into a more

reactive moiety, capable of reacting with some of the functional groups available in the macromolecule. Given that nucleophilic groups are ubiquitously available in peptides and proteins, most of the efforts have historically been directed towards the development of electrophilic mPEG reagents. Moreover, since the structural changes obtained upon modification of the -OH group can be negligible compared with the repeating $(CH_2CH_2O)_n$- unit, huge efforts have been made in order to develop high-yielding polymer activation processes, which ultimately reduces the need for demanding purification steps. Purification and spectroscopic characterization issues of PEG derivatives are beyond the scope of this article, and hence the reader is referred to some excellent review articles [16–19].

The mPEG reagents should be able to react with the functional groups available in the polypeptidic chains under mild (4ºC to room temperature) and buffered aqueous conditions (*p*H from 4.5 to 9, most commonly). Furthermore, the reactivity of mPEG derivatives should be balanced to give acceptable yields of monoconjugated products, but without affording undesirable high degrees of poly-PEGylated bioconjugates, or reacting too quickly with the (aqueous) reaction solvent. In general, most mPEGs are not completely stable and therefore they should be stored in the dark, in the absence of air and moisture.

From early studies, the nucleophilic ε-NH_2 of lysine amino acids has been exploited to anchor electrophilic mPEG reagents to peptides and proteins. Davies and coworkers have shown that the reaction of mPEG cyanuric chloride with bovine serum albumin and bovine liver catalase affords conjugates which conserve 95% of the enzymatic activity, but presents decreased immunogenicity and prolonged circulation half-lives [20,21]. As will be demonstrated later, these PEGylation reactions involve the nucleophilic displacement by the ε-NH_2 group of lysine amino acids on the electrophilic cyanuric chloride-mPEG derivatives. In these PEGylation reactions, high degrees of -NH_2 group modifications were achieved, although with low selectivity.

From the 20 proteinogenic amino acids, only the ones having a suitably-substituted side chain are useful for the attachment of mPEG reagents. In this regard, it is now considered that the most suitable amino acids for PEGylation are: Lys > Cys > Tyr > His > Asp > Glu > Arg > Trp > Ser > Thr >Met. As mentioned earlier, and as will be described in the following pages, the terminal -NH_2 group and ε-amino group of lysine in peptides and proteins are well-established targets for the attachment of different electrophilic mPEG reagents. In fact, these -NH_2 groups together with -SH of cysteine account for the vast majority of the PEGylation reactions. The primary ε-NH_2 group of lysine is a good nucleophile at *p*Hs above 8.5 (*p*Ka 9.4), while terminal amino groups are less reactive, since they are close to the electrophilic carboxyl group (*p*Ka ≈ 1 to 2 *p*H units lower than the ε-NH_2 group of lysine). However, they are able to react with electrophiles at a *p*H of around 7, at which most of the ε-NH_2 groups are protonated. This *p*Ka difference has been used to achieve site-selective PEGylation at the terminal -NH_2 group by reductive amination at slightly acidic *p*Hs. Free -SH groups of cysteines are good nucleophiles, mainly at alkaline *p*Hs. These groups have also been used for the conjugation of proteins to mPEG by means of formation of stable thioether bonds. However, since only a limited number of cysteine residues are usually available in proteins, PEGylation of native -SH groups of proteins was explored to a lesser extent in earlier research.

Conjugation at the carboxyl groups of polypeptides is difficult and has scarce application in PEGylation technology, since activation of this group usually affords undesired protein crosslinking by reaction of the activated carboxylic site with the nucleophilic groups present in proteins. However, this problem has been overcome using mPEG hydrazide under a specific set of reaction conditions [22]. The -OH groups of serine, threonine and tyrosine amino acids, although nucleophilic, are not as reactive as -NH_2 groups towards electrophilic mPEG derivatives. In addition, the reaction of these hydroxyl groups with electrophilic mPEG derivatives can afford ester and carbonate linkages, which are known to be unstable under physiological conditions. The guanidine moiety of arginine is a strongly nucleophilic group whose application in PEGylation with electrophilic mPEG reagents is limited, due to its high *p*Ka; i.e., the conjugation reaction should be performed at high *p*H values in order to have significant amounts of the unprotonated nucleophilic group. However, a site-selective method for the conjugation of arginine has been accomplished recently.

The mPEG reagents are frequently classified in generations, based on their historical appearance, the complexity of the chemistry involved in the synthesis of the mPEG reagent and PEGylation reaction, and the quality of the mPEG starting material.

Thus, the first generation of mPEG reagents was limited to commercially available low molecular weight mPEGs and mPEG raw materials. The PDIs of these mPEG polymers were high, yielding heterogeneous and poly-disperse conjugates. The diol content was also high, which afforded homobifunctional reagents that led to the crosslinking of conjugates and to the formation of inactive and immunogenic protein aggregates. The chemistry required for the synthesis of the reactive mPEG, as well as the PEGylation reactions themselves, was simple and usually limited to very reactive mPEG derivatives, which made the conjugation reactions essentially nonselective, affording heterogeneous and multi-PEGylated conjugates. Many of these reactions involved the formation of hydrolytically unstable linkages, such as ester bonds. Furthermore, these heterogeneous conjugates were often difficult to purify and characterize, hindering batch-to-batch reproducibility. However, the impressive success of the technology allowed for the early success of two PEGylated proteins in reaching the market (Adagen® and Oncaspar®, in 1990 and 1994 respectively, see Table 3.1). Adagen® is a PEGylated form of bovine adenosine deaminase approved by the FDA for the treatment of severe combined immunodeficiency. This conjugate is obtained by random PEGylation with mPEG-succininmidyl ester **1**, with a mass of 5 kDa (Table 3.1). Oncaspar® is a PEGylated form of recombinant asparaginase obtained by random PEGylation with the same reactive mPEG, which is used for the treatment of acute lymphoblastic leukemia [23]. Reagent **1**, as well as other first generation reactive mPEGs, was designed to react with nucleophilic -NH_2 groups by alkylation and acylation reactions. The synthesis of the reagents usually includes simple reaction steps and requires few steps in converting the nonreactive -OH group into electrophilic derivatives.

However, it has been indicated that most of the first generation synthetic methodologies do not work well with high molecular weight mPEGs, affording heterogeneous mPEG reagents that usually contain variable amounts of unreacted polymer, synthetic intermediates or byproducts. In addition, the high diol contents in the

mPEG raw materials yielded high contents of bifunctional polymers, which ultimately led to undesirable crosslinking and inactivation of the proteins. The vast majority of first generation chemistries were directed towards the conjugation of electrophilic mPEG reagents with amino groups and, mainly, with the primary ε-NH_2 group of ubiquitously present lysine amino acids. The improvements achieved in the methods of preparation of mPEG starting materials, as well as the presence of many different companies dedicated to preparing mPEG and mPEG reagents, permitted the easy access to high molecular weight mPEGs with narrow polydispersities and low diol contents. This, together with the huge efforts made in the development of more selective PEGylation reactions, allowed the emergence of a second generation of mPEG reagents that do not exhibit some of the drawbacks associated with the first generation chemistries. Although nowadays nucleophilic amino groups still remain as important targets for the attachment of electrophilic PEGs, some second generation mPEG derivatives were designed to anchor PEG to the thiol group of cysteine amino acids, which are amenable residues for site-selective PEGylation. More recently, several different and promising approaches have been devised to tackle site-selective PEGylation, including enzymatic PEGylation, PEGylation at disulfide bridges, PEGylation at histidine tags and "click" PEGylation of non-natural amino acids, among others [24].

In the following sections some of the most relevant mPEG reagents and PEGylation strategies are presented and discussed. References to the syntheses of the mPEG reagents and the chemistry that support the PEGylation reactions are also provided and discussed. Recent and promising achievements, as well as future expectations are also discussed. New and improved mPEG reagents and strategies are still appearing, many of which are related to previous developments, and thus, classification based on generations can sometimes be confusing. For this reason, and with the aim of providing clarity, PEGylation methods and reagents in this chapter are classified based on the nature of the functional group to which the polymer is attached, and subdivided by the nature of the chemical reaction that supports the PEGylation process.

3.3 PEGylation of Amino Groups

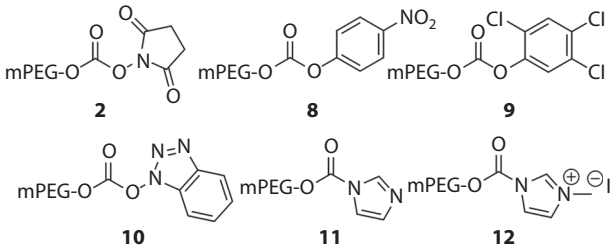
The -NH_2 group of lysine and the terminal -NH_2 group are both well-known targets for the attachment of electrophilic mPEG reagents. Lysine amino acids are widely distributed in several different proteins, which make them particularly useful for conjugation using various mPEG derivatives. The *p*Ka of the ε-NH_2 of lysine is approximately 2 units higher than that of the terminal -NH_2 group, which is translated into the fact that PEGylation of these residues should be performed at slightly alkaline *p*H, usually at around 8-9. As mentioned previously, this *p*Ka difference is exploited nowadays to achieve site-selective PEGylations at the terminal -NH_2 group following a simple reductive amination strategy. PEGylation at nucleophilic -NH_2 groups is usually accomplished by means of acylation (urethane and amide linkages) and alkylation (amine linkage) chemistry.

3.3.1 PEGylation by Urethane Linkage Formation

Electrophilic mPEG reagents **2** and **8**-**12** (Scheme 3.1) are a closely-related family of activated polymer derivatives, which react with primary amino groups of peptides and proteins, affording stable urethane linkages (Equation 3.1).

(3.1)

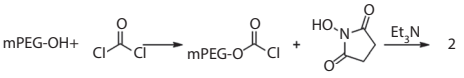
The reactivity of carbonates **2** and **8**-**12** towards the -NH_2 group depends on the nature of the leaving group, which affects not only the selectivity, but also the degree of conjugation. Kinetic data for hydrolysis can be used as an indirect measurement of the nucleophilicity of some of these derivatives, and this data can be found in the literature. Thus, the half-lives of compounds **2** and **10**-**11** are as follows: **2**, 20.4 min (*p*H 8, 25°C); **10**, 13.5 min (*p*H 8, 25°C); **11**, 10 h (*p*H 8, 25°C). Thus, the accepted reactivity order of derivatives **2** and **8**-**11** is: **10**>**2**>**8**>**9**>**11**. Even though the utility of mPEG reagents **2** and **8**-**11** is well documented, activated mPEGs **2** and **10** are currently the reagents of choice for most of the PEGylations involving urethane linkage formation, since they offer a good balance between reactivity and selectivity, but lack the toxicity problems found with reagents **8** and **9**. In fact, reagent **2** is probably the most widely used reagent in PEGylation, and examples of its use are widely reported in the scientific literature. For example, mPEG-succinimidylcarbonate **2** has been employed in the conjugation of several different peptide and proteins, such as asparaginase [25], trypsin [26], chymotrypsin [27], bacteriorhodopsin [28], salmon calcitonin [29], interferon α-2b [30], arginine deiminase [31] and recombinant human arginase I [32], among other biomacromolecules [33]. Reagent **2** is commercially available and can be easily acquired from several different sources. Although derivative **2** reacts mainly with primary -NH_2 groups yielding stable urethane linkages, it has been shown that compound **2** also reacts with the -OH group of tyrosine, threonine and serine to afford hydrolytically unstable carbonate linkages. Depending on the *p*H of the conjugation medium, **2** can also react with the imidazole ring of histidine yielding unstable urethane linkages. The PEGylation of therapeutically important interferon α2-b with **2** has been studied in detail. At *p*H 6.5, activated mPEG **2** reacts with interferon α2-b to give a mixture of conjugates which comprises 47% of PEGylation at histidine residues and 32% at lysine [34]. The bioconjugate obtained under these conditions, employing mPEG of 12



Scheme 3.1 Reagents for the formation of urethane linkages.

kDa, is commercially available (Table 3.1, PEGintron®) and is used for the treatment of hepatitis C and melanoma. PEGylation at histidine residues to yield an unstable urethane bond is also considered as a releasable or reversible conjugation method. In another important application, activated mPEG **2** is also used for the preparation of PEGylated porcine-like uricase, which was recently approved for the treatment of gout (Krystexxa®).

Activated mPEG **2** can be obtained by reaction of mPEG with phosgene, or a phosgene derivative, followed by reaction with *N*-hydroxysuccinimide in the presence of a base, most usually Et_3N (Equation 3.2) [35]. This reaction has been reported to go to completion, providing **2** in almost quantitative yields [36]. A variation of this method, in which toxic phosgene was replaced by the safer triphosgene, has been published in the patent literature [37]. Activated mPEG **2** can also be obtained by the reaction of mPEG with commercially available *bis*-*N*-hydroxysuccinimidylcarbonate **13** using DMAP as base (Scheme 3.2) [38]. Alternatively, the synthesis of **2** by reaction of mPEG with *N*,*N*'-disuccinimidyl oxalate **14** in the presence of pyridine, has also been published (Scheme 3.2). A related reaction, but instead using 1,1'-*bis*[6-(trifluromethyl) benzotriazolyl]oxalate 1**5**, can be used for the synthesis of an activated mPEG closely-related to hydroxybezontriazolyl derivative **10** (Scheme 3.2).

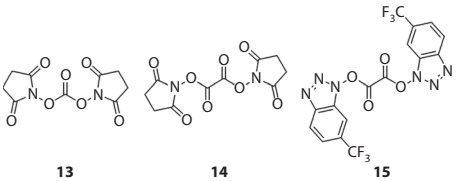


(3.2)

mPEG-4-nitrophenyl carbonate **8** is another important reagent that can be obtained using methodologies similar to those used in the synthesis of **2**. Veronese and coworkers obtained **8** by the reaction of *p*-nitrophenylchloroformate with mPEG in acetonitrile using pyridine as base [39]. Variations of this method include reaction in dichloromethane using either pyridine or triethylamine as base [40,41]. It has been indicated that, in these reactions, variable amounts of an unknown mPEG-amine are also obtained. Pure **8** can be prepared by reaction of mPEG with 4-nitrophenylchloroformate in toluene with tripentylamine [42], or with DMAP in dioxane [43].

Reagent **8** has been used in the PEGylation of ribonuclease and superoxide dismutase [39], doxorubicin [44], recombinant mammalian urate oxidase [45], uricase [46] and lysozyme [47]. However, it has been reported that PEGylations with reagent **8** leave lipophilic toxic impurities on the conjugated material and thus the use of this derivative is now limited.

mPEG **9** was used mainly in early developments. The reagent can be prepared following the same methods as used for the synthesis **8**, but employing commercially available



Scheme 3.2 Reagents for the synthesis of activated mPEGs.

2,4,5-trichlorophenylchloroformate [39]. Although important in early studies, the use of this reagent has almost been abandoned in recent developments.

1-Benzotriazolyl derivative **10** is a relatively new acylating reagent that has found application in the PEGylation of D-glucosamine, sodium heparin, human serum albumin [48], fibrin biomatrix [49], lysozyme [50] and peptides, amongst others [51]. It has been indicated that activated mPEG **10** also reacts with histidine and tyrosine residues to afford unstable urethane and carbonate linkages. Methods for the synthesis of mPEG-benzotriazolyl carbonate **10** can be found mostly in the patent literature, and they essentially involve similar strategies as those presented for the synthesis of **2**, i.e., the reaction of mPEG with phosgene followed by reaction with 1-hydroxybenzotriazole [48], or the reaction with *bis*-1-benzotriazole carbonate in presence of pyridine as base [50].

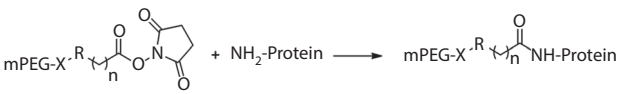
Reagent **11** is a mild PEGylating reagent that has been used since the mid 1980s for the conjugation of proteins, amongst other targets. Due to its low reactivity, activated mPEG **11** is more selective than the other reagents used for urethane linkage formation. In agreement with this reactivity, usually low to moderate yields of conjugation are obtained with reagent **11**, even using large excesses of the reagent and long reaction times. For example, molar excesses of up to 10^4 and reaction times of up to 24 hours can be required to reach moderate yields of conjugation. Various related methods for the synthesis of **11** have been published. Although compound **11** was initially prepared by reaction of mPEG with *N*,*N*'-carbonyl-*bis*-imidazole (CDI) in dioxane at 37°C [52], it was later shown that the yield of the activated polymer obtained following this method was around 42%, probably due to hydrolysis during a long purification step [53]. An improved method that affords activated mPEG **11** with quantitative yields, which involves the use of DMAP in toluene, was later published [53]. Similarly, excellent yields of activation can be achieved for the reaction of mPEG (20 kDa) with CDI in THF at 60°C [54].

Activated polymer **11** is a mild PEGylating reagent that has been applied in pioneering conjugations of different proteins such as superoxide dismutase [52], α2-macroglobulin [55], lactoferrin [52], streptokinase [55], alkaline phosphatase [56], IgG [57] and urokinase [58].

Related to **11**, Vaillard and coworkers have recently prepared the more reactive alkoxy-carbonyl imidazolium iodide **12** and successfully used this reagent for the PEGylation of IFN α-2b [59]. Of note, reagent **12**, which can easily be prepared by alkyation of **11** under mild reaction conditions, affords excellent yields of conjugation even at short reaction times.

3.3.2 PEGylation by Amide Linkage Formation

PEGylation of -NH_2 using activated carboxylic acid esters (Equation 3.3) is a well-developed strategy in bioconjugation technology. In these reactions, the polymer is connected to the activated carboxyl group by means of a linker, the nature of which can have a profound affect on the reactivity of the activated polymer and on the stability of the conjugate.



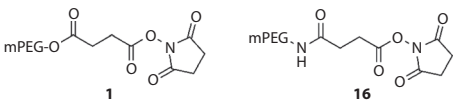
(3.3)

The ease of preparation of mPEG-hydroxysuccinimidyl succinate **1** (Scheme 3.3), resulted in its early use in the conjugation of different proteins. Reactive mPEG **1** proved to be more selective than the alkylating mPEG derivatives that were commonly used by the time this reagent appeared. It has been reported that mPEG **1** reacts selectively with primary amino groups yielding stable amide bonds, showing a half-life of around 20 minutes at *p*H 8 [60].

Succinimidyl succinate **1** is typically prepared by the reaction of succinic anhydride with mPEG under different reaction conditions to give the corresponding succinic acid-terminated polymer, followed by diimide-mediated esterification with *N*-hydroxysuccinimide [61]. The DMAP accelerated ring-opening reaction as well as melt synthetic method have also been published [62].

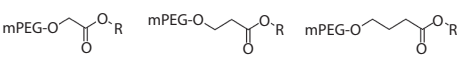
The ester linkage between the polymer chain and the succinate moiety is very sensitive to hydrolysis under physiological conditions. Thus, the linkage between the acid moiety and the polymer can be lost due to hydrolysis of the ester bond. In fact, the conjugation of activated **1** with proteins is recognized as an early example of releasable PEGylation. Given that the succinate moiety remains linked to the protein after hydrolysis, and that this group can act as a hapten, increasing the immunogenicity of the protein [63], the actual trend is to use other PEGylation reagents to achieve amide linkage formation. Related reactive mPEGs on which the polymers are linked to the succinic acid residue by stable amide bonds, such as **16**, are also well known (Scheme 3.3) [17]. In a related approach, mPEG can be attached to different amino acids, by means of stable urethane bonds, using activated mPEGs, like reagent **2** [64]. In this regard, the activated mPEG bearing a norleucine moiety is particularly important since it can be used to study the degree of PEGylation by amino acid analysis of the mPEG-protein conjugates [65]. Other amino acids that have been used as linkers between mPEG and proteins include ^{14}C- or tritium-labelled glycine, tryptophan and β-alanine, which have been employed for the bioconjugation of proteins for different applications. In addition, Met-NLe and Met-βAla di-peptides were used for the identification of PEGylation sites [66].

As indicated previously, the ease of preparation of reactive mPEG **1** stimulated its evaluation for conjugation to several different proteins. The most relevant applications of **1** involve its use in the preparation of commercially available Adagen® (adenosine deaminase [35], Table 3.1) and Oncaspar® (asparaginase [67], Table 3.1). Other interesting examples include the PEGylation of peptides [68], alkaline phosphatase [56], rHuG-CSF [69], arginine deiminase [70] and other proteins [71].



1 **16**

Scheme 3.3 Activated mPEGs for amide linkage formation.

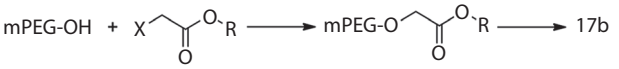


17a; R: NHS **17b;** R:H **18a;** R: NHS **18b;** R:H **19a;** R: NHS **19b;** R:H

Scheme 3.4 Caboxylic acid-terminated mPEGs.

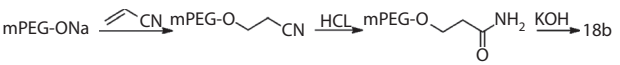
Activated polymers **17a**-**19a** (Scheme 3.4, where NHS = *N*-hydroxysuccinimidyl) are a closely-related family of mPEG derivatives to **1**. In these reagents the polymers are attached to the spacers by hydrolytically-stable ether linkages. As indicated for activated ester **1**, compounds **17a**-**19a** also react with primary -NH_2 groups, such as the ε amino group of lysine, affording stable amide bonds (Equation 3.3). Reactive esters **17a**-**19a** are obtained by esterification with *N*-hydroxysuccinimide of mPEG-acetic (**17b**), mPEG-propionic (**18b**) and mPEG-butyric acid (**19b**), respectively (Steglich-type esterifications). Although structurally simple, the preparation of these widely used activated mPEGs is usually not facile, and given their utility for the PEGylation of therapeutically relevant proteins, most of the methods for syntheses of **17**-**19** have been published in the patent literature.

mPEG-acetic acid ester **17a** has been employed in PEGylation technology. The synthesis of the parent mPEG-acetic acid **17b** by direct oxidation of mPEG with $KMnO_4$ does not work well since polymer chain degradation is usually observed. Alternatively, a more selective stepwise oxidation with MnO_2, followed by H_2O_2, should be used for the synthesis of **17b**. However, many of these simple oxidations tend to fail with high molecular weight polymers. Thus, a straightforward and more convenient synthesis of mPEG-acetic acid **17b** has been proposed, which involves the reaction of mPEG alkoxide with alkyl 2-haloacetate, followed by hydrolysis under either acidic or alkaline conditions (Equation 3.4). The mPEG alkoxides can be obtained by acid-base reaction of the -OH of the polymer with sodium or potassium *tert*-butoxide, sodium naphthalenide, NaH or butyllithium [17]. Most often, mPEG alkoxides are obtained by an acid-base reaction with *tert*-butoxide in *tert*-butanol, and then reacted with *tert*-butyl 2-bromoacetate (Equation 3.3, where X = Br and R = ^{t}Bu). Although the hydrolysis of the intermediate mPEG-ester may be done with trifluoroacetic acid, it has surprisingly been claimed that the hydrolysis step can be best performed under standard basic conditions, which prevents the degradation of the polymer chain. For this method, yields of up to 100% have been reported [72].



(3.4)

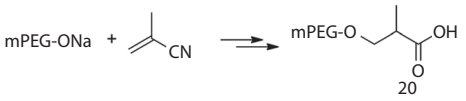
mPEG propionic acid **18b** has been prepared by conjugate addition of mPEG alkoxide to acrylonitrile, followed by hydrolysis in strongly basic conditions [73]. Using mPEG of 20 kDa it was shown that this method affords 68% of the required polymer derivative (Equation 3.5).



(3.5)

A detailed study for the synthesis of **18b** using acrylonitrile as the Michael acceptor has been performed by Harris and coworkers using mPEG of 2 kDA. They have shown that the addition reaction can be performed in water using potassium hydroxide as base and that the hydrolysis sequence is key to obtaining good yields of mPEG-propionic acid. It has also been indicated that the first hydrolysis step to yield the intermediate amide is best performed with alkaline H_2O_2, which in addition prevents polymer chain degradation [74].

In a related work, but employing α-methylacrylonitrile as Michael acceptor, α-methylpropionic mPEG acid derivative **20** was obtained (Equation 3.6) [75,76].

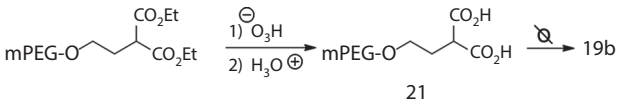


20 (3.6)

A related method for the synthesis of **18b**, which involves the use of *tert*-butyl acrylate as an electrophilic acceptor, has been reported in the patent literature. It has been suggested that this method works well with high molecular weight mPEGs (20 and 30 kDa (68%)) and it was indicated that the reaction does not proceed in the absence of the quaternary ammonium salt as a phase transfer catalyst [77].

It has been published and patented that mPEG-propionic acid can be obtained by nucleophilic displacement reaction of mPEG-alkoxide with ethyl 3-bromopropionate or with halogenated 3-halopropionate substrates [78,79]. However, it was later indicated that the first method does not afford the required acid but the elimination product, due to the acid-base reaction of the hydrogen in the position α– to the carboxylic group [74].

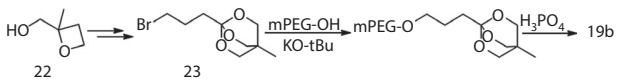
mPEG-butyric acid **19b** has been prepared by a nucleophilic substitution reaction using diethylmalonate anion and mPEG-methanesulfonate (mPEG-mesylate) as substrate, yielding di-ester **21** (Equation 3.7). Hydrolysis in alkaline media followed by decarboxylation furnished the target mPEG-acid derivative **19b** (Equation 3.7) [72].



21 (3.7)

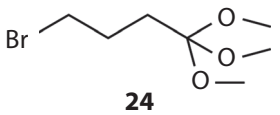
Furthermore, the synthetic strategy depicted in Equation 3.7 but using 2-methyldiethylmalonate anion, has also been used for the preparation of mPEG-2-methylbutyric acid, which was suggested to behave as a mild PEGylation reagent.

Other interesting syntheses of mPEG butyric acid have also been published in the patent literature [80]. For example, ortho-ester **23** can be obtained by a two-step procedure from 4-bromobutanoyl chloride and 3-methyl-3-oxetanemethanol **22** (Equation 3.8). A nucleophilic substitution reaction of **23** with mPEG-alkoxide, followed by hydrolysis, gives the required acid **19b** (Equation 3.8). The decrease in the acidity of the proton α- to the now protected carbonyl, removes the possibility of elimination reactions observed with 3-bromopropionate and related halo-esters.



22 23 (3.8)

Commercially available trimethyl 4-bromo*ortho*butyrate **24** can be used as an equivalent electrophilic starting material in a similar substitution reaction (Scheme 3.5).



24

Scheme 3.5 4-bromo*ortho*butyrate **24.**

In addition, it has been shown that nucleophilic substitution reactions of mPEG-alkoxides with ethyl 5-bromovalerate and ethyl 4-bromo-4-methyl butyrate, yield mPEG-ethyl valerate and mPEG-ethyl 2-methyl butyrate respectively, which upon hydrolysis should afford the corresponding free acids [81,82]. Presumably these reactions also give elimination products, as indicated for ethyl 3-bromopropionate.

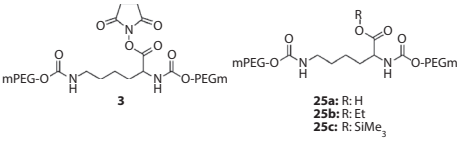
It is well known that the reactivity of activated mPEG acids **17a**-**19a** towards the -NH_2 group of peptides and proteins depends on the length and the presence of substituents in the hydrocarbon spacer moiety. Thus, the longer the hydrocarbon chain between the polymer and the reactive center, the lower the reactivity towards the amino group and, hence, the higher the selectivity of the PEGylation reaction. The hydrolysis rates of derivatives **17a**-**19a** and of *N*-hydroxysuccinimdyl esters of **20** and of mPEG-4-methyl butyric acid and related compounds correlate well with this reactivity trend. For example, the half-lives of the previously mentioned activated esters are 0.75, 16.5, 23.3, 33 and 44 minutes respectively (25°C, *p*H 8) [73,77].

mPEG-acetic acid **17a** and mPEG-propionic acid derivative **18a** have been intensively employed in bioconjugation of relevant proteins, such as methioninase [82], insulin [83], murine GM-CSF [84], glucagon-like peptide 1 [85], protamine [86], salmon calcitonin [87], α-lactalbumin [88], recombinant human arginase I [32], concanavalin A [89], pretreatment of allografts [90], adenovirus vectors [91,92], platelet CD42a [93], red blood cells [94], formate dehydrogenase [95] and antibodies against glutamic acid decarboxylase [96–98].

An example of the use of activated mPEG-butyric acid **19a** includes the PEGylation of erythropoietin β [99]. This conjugate, commercially available as Mircera®, is used for the treatment of anaemia associated with chronic kidney disease.

Branched mPEGs bearing activated proximal carboxylic groups are particularly well suited for the improvement of the biopharmaceutical properties of several therapeutically relevant proteins. Branched mPEGs behave as if they have higher molecular weights than linear mPEGs of identical molecular weights. Moreover, if branched mPEGs are used, more than one polymer chain can be linked into each attachment site. The result of these two effects is reflected in a better shielding of the protein, while diminishing the possibility of deactivation of the active site of the protein. In particular, branched mPEGs bearing a lysine linker **3** (Scheme 3.6, Table 3.1) proved to be particularly suitable for the conjugation of several different proteins such as: ribonuclease, catalase, asparaginase, trypsin [100], interferon α-2a [101–103], interferon α-2b [104], interferon β-1b [105], TNYL-RAW – Fc portion of human IgG1 [106], peptides targeting the human neonatal Fc receptor [107], α-momorcharin [108,109], erythropoietin [110], organophosphorus hydrolase [111] and lactoferrin [112], among several other examples.

Various different methods for the synthesis of **3** can be found in the literature. For example, the first reported synthesis of **3** involved the reaction of mPEG-*p*-nitrophenylcarbonate **8** with lysine in buffered aqueous media, affording intermediate



Scheme 3.6 Branched mPEGs bearing a lysine linker.

mPEG-monosubstituted lysine. This mono-conjugated product was then further treated with the same reagent in methylene chloride to yield **25a**, which can then be converted to **3** by diimide-mediated esterification with *N*-hydroxysuccinimide [113]. This two-step procedure allows for the preparation of branched **3**-bearing mPEG chains of different molecular weight [100]. An easier method for the synthesis of **3** was also introduced by the same authors. Thus, the reaction of mPEG-*N*-hydroxysuccinimidyl carbonate **2** with lysine in buffered aqueous solution afforded branched **25a** in a single step [100]. In these methods, **25a** was purified by ion exchange chromatography. The related **25b** has been prepared by reaction of lysine with **2** using pyridine as base. Hydrolysis of ester **25b** followed by activation under standard conditions provides **3** [114].

We have recently introduced a simple and straightforward method for the synthesis of **3**. *N*-Carbamoylimidazolium iodide **12** was obtained in high yield by a simple two-step procedure and then reacted with an organic solvent soluble silylated lysine derivative to yield **25c** in a single step. Compound **25c** was converted to **25a** during the aqueous work-up of the reaction [54].

An elaborate but useful method for the preparation and use of **3** in the PEGylation of proteins that avoids intermediate purification steps has been published in the patent literature [37]. A noteworthy point is that only one purification step is performed after the conjugation reaction.

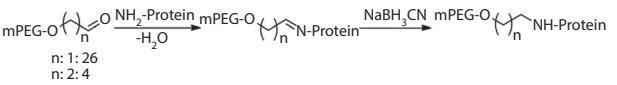
The relevance of reagent **3** (or of intermediate **25a**) is clear when it is considered that four out of the eleven PEGylated products currently available on the market contain the branched mPEG2Lys structural motif (Table 3.1, Pegasys® (interferon α-2a), Macugen® (aptanib), Cimzia® (anti-TNF fab') and Omontis® (dimeric synthetic peptide for the stimulation of erythropoiesis).

In connection, a new tri-branched mPEG3Lys2 has recently been successfully employed for the conjugation of interferon α-2 [115].

3.3.3 PEGylation by Reductive Amination

It is well known that under slightly acidic conditions (*p*H 4.5–5.0) carbonyl groups react with nucleophilic amines to yield Schiff bases that can be isolated or further converted to the corresponding amines by reaction with an adequate reducing reagent, such as $NaBH_4$ or LAH. In PEGylation technology, the reductive alkylation reaction has successfully been used to achieve mild and site-selective conjugation reactions using mPEG-acetaldehyde **26** and mPEG-propanaldehye **4** with $NaBH_3CN$ as the reducing reagent (Equation 3.9). Although $NaBH_3CN$ is usually the reducing reagent of choice, it has recently been suggested that other boranes, such as pyridine- and 2-picoline borane, can be used in PEGylations proceeding by

the reductive amination method [116]. mPEG-propanaldehyde **4** is better suited for PEGylation than acetaldehyde **26** since the latter is unstable and dimerizes by aldol condensation reaction.

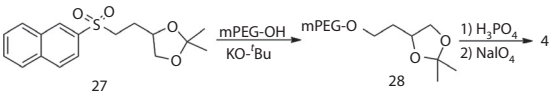


(3.9)

In the PEGylation of -NH_2 groups with electrophilic reagents **26** and **4**, different issues should be considered: i) the nucleophilicity of the particular -NH_2 group involved in the condensation reaction, and ii) the *p*H at which the condensation reaction is performed. As indicated previously, the ε-NH_2 group of lysine is more nucleophilic and basic than most terminal (and α carboxyl) -NH_2 groups. Under the slightly acidic conditions required for the condensation reaction, the more reactive ε-NH_2 groups are in the protonated and non-nucleophilic form, allowing site-specific PEGylation at the terminal -NH_2 group. The reductive amination reaction can be seen as a two-step alkylation reaction, and thus, the net charge of the protein does not change after the conjugation reaction.

The synthesis of mPEG-acetaldehyde **26** by direct oxidation of mPEG is not simple. For example, it has been indicated that the oxidation of mPEG with pydrinium chlorochromate in methylene chloride, affords **26** in low yield (30%) [117]. Using mPEGs of 20 kDa, we have found that Dess-Martin periodinane is useful for the preparation of **26** giving the required aldehyde in high yield [118]. Aldehyde **26** can also be prepared by a Moffat-type oxidation reaction or by the nucleophilic substitution reaction of mPEG potassium alkoxide with chloro- or bromoacetaldehyde diethylacetal, followed by acid hydrolysis [119–121]. The last method has been proposed as the method of choice for the synthesis of **26**. In a similar approach, aldehyde **4** can be obtained by nucleophilic substitution of mPEG alkoxide with 3-chloropropanaldehyde diethyl acetal, under several different conditions, followed by deprotection (50%) [122]. On the other hand, it has been indicated that this reaction affords a mixture of substitution and elimination products. A detailed kinetic analysis showed that 15 equivalents of the substrate in boiling dioxane or toluene are the optimal experimental conditions to obtain the required protected aldehyde **4** with quantitative yield [123].

Other methods for the preparation of **4** have been published, mainly in the patent literature. For example, it has been claimed that 2-naphthalenesulfonyl chloride reacts with 2,2-dimethyl-1,3-dioxolane-4-ethanol, yielding synthetic intermediate sulfonate **27** (Equation 3.10). Sulfonate **27** was then reacted with the mPEG alkoxide to afford a protected mPEG-diol derivative **28,** which after hydrolysis under acid conditions, followed by oxidation with $NaIO_4$, gave mPEG-propionaldehyde **4** in good yield (Equation 3.10) [124].

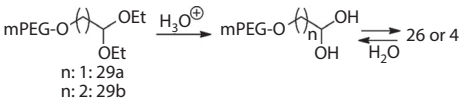


27 28

(3.10)

Diethylacetals **29a** and **29b** are more stable and can be obtained with higher purities than the mPEG-aldehydes **26** and **4**. Hence, it has been indicated that **29a** and **29b** are

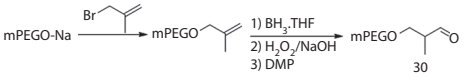
better suited for the use in PEGylations proceeding by the reductive amination strategy. The diethylacetals **29a-b** are easily transformed to the aldehyde hydrates under acidic conditions. Thus, after adjusting the *p*H, they can be directly used for the conjugation reaction (Equation 3.11) [125].



(3.11)

Several different proteins have been PEGylated with aldehydes **26** and **4**. Some interesting examples include the PEGylation of horseradish peroxidase [126], CD4 immunoadhesin [119], doxorubicin [127], recombinant human granulocyte colony-stimulating factor (Filgrastim, r-metHuG-CSF) [128,129], human megakaryocyte growth and development factor (MGDF) [130], lysozyme [131], tumor necrosis factor receptor type I [132], interferon β-1a [133], octreotide [134], recombinant human arginase I [32], recombinant erythropoietin [110], porcine follicle-stimulating hormone [135] and others [122,124].

Baker and coworkers introduced 2-methylpropionaldehyde mPEG derivative **30** and successfully used this reagent for the site-selective conjugation of interferon β-1a. mPEG aldehyde **30** can be easily prepared by a three-step procedure that affords the reagent in excellent yield (Equation 3.12) [136].

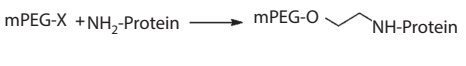


(3.12)

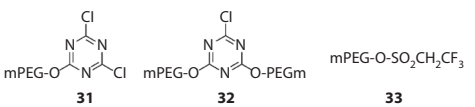
Noteworthily, this conjugate has completed phase 3 clinical trials and has been submitted for FDA approval for the treatment of multiple sclerosis (Plegridy®).

3.3.4 PEGylation by Alkylation

The pioneering work of Abuchowski on PEGylation was based on alkylation reactions using extremely reactive mPEG-dichlorotriazine **31** (Scheme 3.7). This compound reacts with nucleophilic -NH_2 groups yielding stable amine linkages (Equation 3.13) [20,21]. As with the reductive amination process, alkylation reactions give conjugates that preserve the same charge as the native protein.



(3.13)



Scheme 3.7 mPEGs reagents based on tri-chlorotriazine and tresylate.

It has later been shown that reagent **31**, which can be obtained by the reaction of mPEG or mPEG alkoxide with cyanuric chloride, reacts in a nonselective fashion with the nucleophilic groups present in proteins, such as lysine, tyrosine, histidine, cysteine and serine. PEGylation reactions with **31** and related branched compound **32** are performed at neutral or basic *p*H (7.4–10.0).

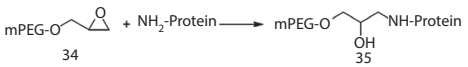
After the first substitution, the remaining chloride of **31** is reactive enough for a second substitution, and this can lead to undesirable crosslinking of the proteins. Several examples of the use of **31** have been published, mainly in early studies, including the PEGylation of ovoalbumin [137,138], bovine serum albumin [20], superoxide dismutase [139], elastase [140], acyl-plasmin-streptokinase complex [141], trypsin [142], silk fibroin [143] and immunoglobulin [144], catalase [145,146], amongst others.

The Y-shaped branched mPEG **32** is more suitable than **31**, given that the decreased reactivity obtained upon replacement of two chlorine atoms yields milder and more selective PEGylation reagents [147]. In addition, since **32** has only one leaving group, protein crosslinking is not possible. Moreover, reactive mPEG **32** has two polymer chains linked to one reagent molecule, allowing the attachment of two mPEG chains on each conjugation site, decreasing the possibility of inactivation of the biomacromolecule activity (Equation 3.6). Branched polymer **32** can be obtained by the reaction of mPEG with cyanuric chloride in benzene using Na_2CO_3 as base. The activated polymer obtained in this way was successfully used in the conjugation of *E. coli* asparaginase [148]. It was later demonstrated that this synthetic method affords a mixture of linear and branched polymers **31** and **32**, which ultimately led to complex mixtures of PEGylated products. An improved method that affords **32** as a single product has also been published [149]. Further examples of the use of branched mPEG **32** include the PEGylation of ovoalbumin and phenylalanine ammonia-lyase [150,151].

mPEG tresylate (mPEG-2,2,2-trifluoroethanesulfonate) **33** is another well-known reactive derivative that has been employed as an electrophilic mPEG reagent for the conjugation of proteins by mean of alkylation reactions. Activated mPEG **33** can easily be prepared by the reaction of mPEG with tresyl chloride in the presence of pyridine [152]. In the same fashion as with reagents **31** and **32**, PEGylation of peptides and proteins with **33** is performed under mild conditions, furnishing conjugates that retain the net charge of the unmodified biomacromolecule [153]. A detailed kinetic study on the coupled hydrolysis-nucleophilic substitution reaction of PEG *bis*-tresylate with amines has been performed by Griffith and coworkers [154]. It was suggested that low temperatures and a *p*H of around 8.0 are the best conditions for the conjugation of secondary -NH_2 groups, while the *p*H should be increased to around 8.8 for the conjugation to the ε-NH_2 amino group of lysine. In addition, it has also been indicated that **33** reacts in a non-specific fashion with various nucleophilic groups present at the aminoacids side chains, giving complex mixtures of products that can involve unstable bonds, resulting in poorly defined mixtures of conjugated adducts [155]. For this reason, the use of mPEG **33** has been almost abandoned. Representative proteins that have been conjugated to mPEG using tresylate **33** include lipases [156], alkaline phosphatase [157], albumin [152], granulocyte-macrophage colony-stimulating factor [158], insulin [159] and B-deleted recombinant coagulation factor VIII [160]. Related mPEG tosylates are also well-known activated polymers that have been used, for example, in the PEGylation of trypsin [161]. In connection, mPEG-mesylate, mPEG-bromide and mPEG-chloride

are all well-known electrophilic derivatives. These compounds have mainly been used as synthetic intermediates for the preparation of more elaborate reagents.

mPEG-epoxide **34** has been used as a mild PEGylating reagent that reacts with -NH_2 groups under mild conditions (Equation 3.14). Reagent **34** has found application mainly in material and polymer sciences, among other uses [162–166]. mPEG derivative **34** reacts with -NH_2 groups of peptides and proteins producing stable secondary amine bonds (conjugate **35**, Equation 3.14). Due to the low reactivity of this electrophilic mPEG reagent, its application for the PEGylation of biomacromolecules has been limited. In addition, it has been suggested that this reagent is not selective for -NH_2 group conjugation, since it reacts with the -OH, -SH and imidazole groups. Examples of the use of **34** include the PEGylation of glutathione, bovine serum albumin, protein A, and monoclonal antibodies [167–169].



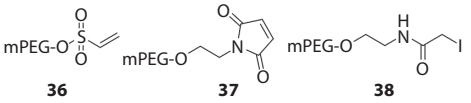
(3.14)

Epoxide **34** can be prepared by nucleophilic reaction of mPEG with the oxirane ring of epichlorohydrin in the presence of $BF_3.Et_2O$ complex. The intermediate alcohol product is then dehalogenated with sodium aluminate to afford the required mPEG reagent **34** [170].

3.4 PEGylation of Thiol Groups

In the last two decades different technologies have been developed with the aim of tackling site-selective PEGylation. In this regard, PEGylation at -SH group has been the object of intense research in academia and in the industry. Cysteine amino acids are scarcely present in peptides and proteins. In addition, cysteine amino acids are hydrophobic and they are usually located in the internal part of proteins, limiting their availability for conjugation. On the other hand, -SH group PEGylations at cysteine residues located in the surface of the protein usually do not compromise the protein bioactivity, taking full advantage of the benefits of PEGylation. The -SH moiety is one of the most reactive nucleophilic groups present in biomolecules, which can react with electrophilic mPEG reagents at slightly acidic or neutral *p*Hs (6-7). At these *p*H values nucleophilic -NH_2 usually cannot compete with -SH nucleophiles.

Importantly, genetic engineering methods are available nowadays for the introduction of cysteine residues in peptides and proteins; and this approach has been employed for the PEGylation of proteins whose native forms do not have these amino acidic residues. [171,172]. Although useful, this strategy can steer to the formation of unwanted disulfide linkages and to protein dimerization. Furthermore, another approach that has been developed to increase the amount of available free cysteine amino acids involves the reduction of protein disulfide bridges using adequate reagents [173]. This method has found particular application for the site-selective PEGylation of antibodies, whose activity can be critically decreased by terminal -NH_2 group conjugation. Of course, these conjugation methods are site-selective when the protein possesses only one disulfide bridge, or when only one of two or more of the disulfides bridges can be reduced selectively.

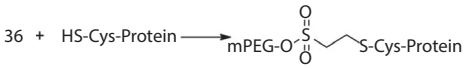


36 **37** **38**

Scheme 3.8 mPEG reagents for the conjugation of -SH group.

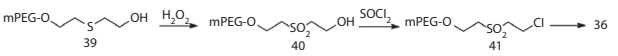
From all commercially available reagents that are capable of reacting selectively with the -SH groups, most of the attention have been centered on mPEGs **36**-**38** (Scheme 3.8).

Vinyl sulfone **37** reacts selectively with -SH group of cysteine in slightly basic conditions in Michael-type addition reaction to afford stable thioether linkages (Equation 3.15). It has been indicated that lysine conjugation also occurs at more basic *p*H (*p*H 9.3) when large excesses of **36** are used.



(3.15)

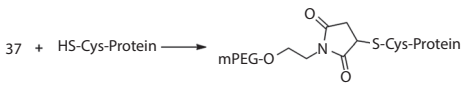
mPEG-vinylsulfone **36** was prepared following the reaction sequence presented in Equation 3.16. Mesylation of mPEG under standard conditions, followed by nucleophilic substitution with 2-mercaptoethanol under basic conditions affords sulfide **39** (Equation 3.16). Oxidation with H_2O_2 yields sulfone **40**, which was then reacted with $SOCl_2$ to give chloride **41**. Finally, chlorosulfone **41** was dehalogenated to the target reagent in quantitative yield by a base-promoted elimination (Equation 3.16) [174].



(3.16)

A simple one-step "click" method for the preparation of vinyl sulfone **36**, among other hydroxyl-terminated polymers, has been published. In this method, *bis*-vinyl sulfone is employed, which upon reaction with mPEG-alkoxide gives **36** in a single step and with high yield [175].

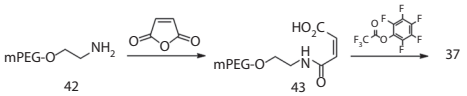
mPEG-maleimide **37**, and other closely related compounds, are well-known reagents that have intensively been used to achieve site-selective conjugation at cysteine amino acids. In the same fashion as with **36**, reagent **37** reacts with the -SH group in a Michael-type addition reaction, yielding hydrolytically stable thioether linkages (Equation 3.17). As observed for **36**, with reagent **37** side-reactions which arise from the conjugation with $-NH_2$ groups, are also produced, mainly at alkaline *p*Hs, although at very low rates. Moreover, it has been indicated that the rate of $-NH_2$ conjugation increases in the presence of an organic co-solvent [64].



(3.17)

The synthesis of mPEG reagent **37** has been published in the patent literature as depicted in Equation 3.18. Tosylation of mPEG under standard conditions, followed by nucleophilic displacement reaction with aqueous ammonia solution gives mPEG-amine **42**. Acylation

of **42** with succinic anhydride yields unsaturated amide derivative **43**, which can then be converted to the target maleimide under harsh reaction conditions (Equation 3.18) [176].



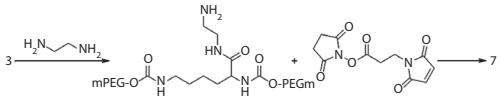
(3.18)

Although other methodologies for synthesis of **37** have been published, it has been indicated that these methods afford undesirable high amounts of byproducts [177–179].

More recently it has been claimed that mPEG-amine **42** can react with *N*-methoxycarbonylmaleimide in aqueous basic solution, rendering the required mPEG maleimide **37** in a single step [180].

Reagent **37** is one of the most widely employed mPEGs for site-selective conjugation of -SH groups. Compound **37** has been used, for example, in the PEGylation of interleukin-2, staphylokinase, rHuGM-CSF, erythropoietin analogues, cyanovirin–N, prolidase, recombinant human arginase I, si-RNA, bone morphogenetic protein-2, exenatide analogues and aptamers, amongst many others [32,181–193]. A drawback found in the use of this derivative relates to the instability of the reagent.

Branched $mPEG_2$-lysyl-maliemide **7** (Table 3.1) is a useful PEGylation reagent that has found application for the conjugations of antibody fragments. A remarkable application of **7** involves its use in the preparation of Cimzia® (Table 3.1). Maleimide **7** can be obtained starting with branched succinimidyl ester **3** by a two-step method (Equation 3.19) [194].

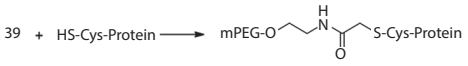


(3.19)

Further examples of the use of **7** in the PEGylation of macromolecules are well documented in the literature [195,196].

Other mPEG reagents bearing activated unsaturated bonds, such as mPEG acrylate, have also been used as Michael acceptor for selective cysteine PEGylation [197].

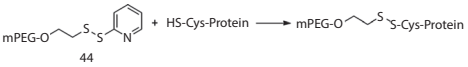
mPEG-iodoacetamide **38** is a mild PEGylating reagent that reacts with the -SH via nucleophilic substitution reaction yielding stable thioeter linkages (Equation 3.20). As mentioned for derivatives **37** and **38**, some loss in the selectivity of the conjugation reaction is observed as the *p*H is increased.



(3.20)

The PEGylation reactions with **38** are usually performed using an excess of the reagent in a darkened flask, in order to limit the formation of iodine, which can react with other amino acids. mPEG-iodoacetamide is a commercially available reagent that can be prepared by the reaction of mPEG-amine **42** with iodoacetic anhydride in dioxane [178]. Examples of the use of **38** include the PEGylation of acyl-coenzyme A and cholesterol acyltransferase [198].

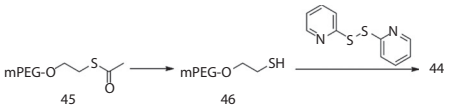
mPEG-*ortho*pyridyldisulfide **44** (mPEG-OPSS) is another useful reagent for -SH site-selective PEGylation. This compound reacts with -SH groups in acid and alkaline media via a thiol exchange reaction to afford unstable disulfide linkage (Equation 3.21), which can be broken under reducing physiological conditions.



(3.21)

Several other mPEG-OPSS derivatives having different spacers between the polymer chain and the pyridyl-disulfide moiety have been patented [199].

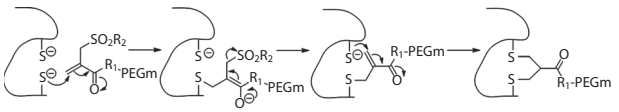
mPEG-OPSS **44** has been prepared by tosylation of mPEG followed by nucleophilic displacement with potassium thioacetate to give intermediate thioester **45**, which after methanolysis and treatment with dithiothreitol yielded mPEG-thiol **46** (Equation 3.22). Finally, the reaction of the free thiol group of **46** with *ortho-bis*-dithiopyridine affords sulfide **44** (Equation 3.22) [200].



(3.22)

Examples of the use of reagent **44** include the PEGylation of interferon β and staphilokinase [182,200,201].

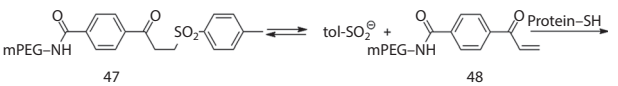
Chemically accessible disulfide bridges are amenable for site-specific conjugation. Thus, in TheraPEG® technology, disulfide bridges are reduced under mild conditions to give the free -SH groups (Equation 3.23), which are then trapped by a double Michael-type 1,4-addition using an unsaturated sulfone as acceptor, affording a stable cyclic bisulfide linkage (Equation 3.23) [173].



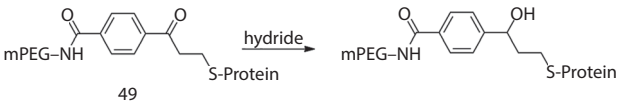
(3.23)

Noteworthily, it has been demonstrated that the conjugation reaction does not affect the tertiary structure of the protein and, importantly, that, due to steric hindrance at the conjugation site, only one polymer chain is attached at each disulfide bridge.

Recently, Brocchini and coworkers further expanded this concept and designed mono-sulfone **47** for site-selective PEGylation of -SH groups [202]. Mono-sulfone **47** behaves as a latent PEGylating reagent, which upon elimination of tolyl-sulfonyl anion generates unsaturated ketone **48**, which reacts in Michael-type addition reaction with -SH to afford conjugate **49** (Equation 3.25). To avoid retro-Michael addition, the electrophilic carbonyl group should be reduced using a suitable hydride to give stable -OH group (Equation 3.25). Moreover, it was demonstrated that the conjugates obtained with reagent **47** are more stable than those obtained with mPEG-maleimide derivatives.

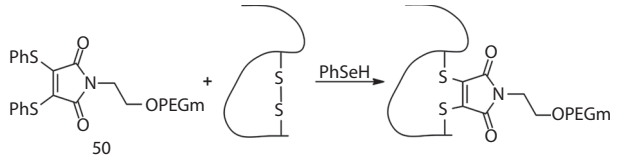


(3.24)



49 (3.25)

The PEGylation of -SH groups employing dithiophenolmaleimides **50** has been reported. The free -SH groups, generated upon reduction with a suitable reagent, are quickly trapped by **50** (Equation 3.26). It has been suggested that using this method it is possible to restrict the unfolding, aggregation and disulfide scrambling of the protein [203]. A closely related method that uses dibromomaleimide derivatives has also been developed [204].



50 (3.26)

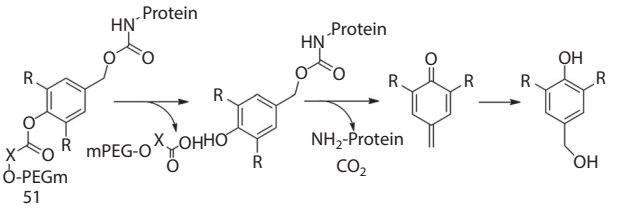
3.5 Reversible PEGylation

As described previously, the covalent attachment of mPEG has a profound effect in the biopharmaceutical properties of peptides and proteins. Moreover, it is usually regarded that the linkage between the polymer and poly-peptidic chain should be stable in physiological conditions, in order to allow an increment of the half-life of the conjugate. On the other hand, nowadays it is well known that the attachment of the polymer at, or close to, the active site can lead to drug inactivation by steric hindrance. With the aim of surpassing drug inactivation and loss of the biological activity, several different approaches for releasable or reversible PEGylation have been developed in the last years, which have found application, mainly, for the controlled delivery of small drugs. [205,206]. In releasable PEGylation, the polymer is bound to the bioactive molecule through an unstable bond or a degradable linker which can be cleaved under specific conditions, releasing the native unmodified drug at a controlled rate. In fact, in releasable PEGylation the drug-polymer conjugate is seen as a prodrug, from which the unmodified active drug is delivered after polymer release. In the particular case of peptides and proteins, the PEGylated prodrug can show reduced activity or be completely inactive. Moreover, the PEGylation of hydrophobic small drugs with this hydrophilic polymer has been used to increase the solubility, and hence for the delivery of poorly soluble drugs.

Several different unstable or degradable linkages have been used in reversible PEGylation, including esters, carbonates, carbamates, hydrazones and amides, among others. As indicated previously, these approaches proved to be particularly useful for the conjugation of small drugs. Successful examples of the use of PEGylation for controlled delivery include the conjugation of paclitaxel, camptothecin and podophyllotoxin, and various others [205,207,208]. The design of the linker which brings together the protein and the polymer chain, forming a tripartate prodrug, is a key issue that determinates the mechanism of the release, in this way governing the rate of delivery.

Reversible PEGylation reactions involving 1,4- or 1,6- benzyl elimination (BE) and bicine linkers are well-established strategies for releasable PEGylation of small drugs,

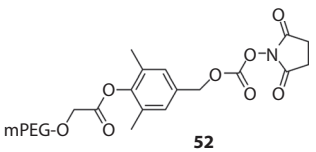
such as daunorubicine, vancomycin, amphotericine B, and doxorubicine [209–212]. Furthermore, benzyl elimination has been found to also be well suited for reversible PEGylation of proteins. This approach is based on the initial cleavage of an hydrolytically unstable polymer-linker bond from bioconjugate **51**, after which a well-known fast 1,4- or 1,6-elimination delivers the unmodified native protein (Equation 3.27) [213,214].



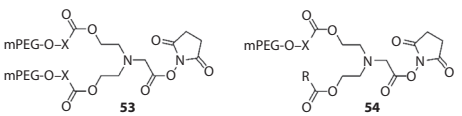
(3.27)

The chemistry of the PEGylation reaction with the linker involves the formation of urethane linkages with free -NH_2 groups of the biomacromolecule. The mPEG reagents used in this approach usually are *N*-hydroxysuccinimidyl carbonates, which react with proteins in the same fashion as indicated in Equation 3.1. One important advantage of the benzyl elimination approach is that the kinetics of the release can be finely tuned by the introduction of substituents of different natures and sizes in the *ortho* position to the -OH group (R substituents in conjugate **51**). Moreover, the nature of the bond that brings together the polymer and the linker (trigger group) can also be modified, providing further possibilities for the fine control of the delivery. It has been shown that the nature of the trigger groups may also have an influence in the reactivity of the PEGylation reagent. Thus, reagent **52**, which has two *ortho* methyl groups and the mPEG chain linked by an ester moiety, is particularly attractive for releasable PEGylation (Scheme 3.9).

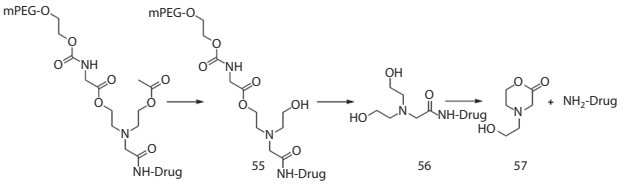
Branched and linear bicine linkers **53** and **54** have found application for the controlled delivery of small drugs and proteins (Scheme 3.10) [210,215]. The chemistry of the delivery using a linear derivative is illustrated in Equation 3.28. Hydrolysis of the aliphatic ester group affords hydroxyethyl intermediate **55**. Intermediate **55** reacts with water at a controlled rate rendering *bis*-hydroxyethyl intermediate **56**, which undergoes a well-known cyclization reaction to give bicine **57** and delivering the native drug or protein (Equation 3.28).



Scheme 3.9 Reagent **52**, useful for releasable PEGylation by 1,4- or, 1,6-BE.



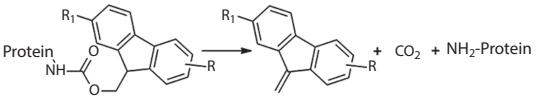
Scheme 3.10 Bicine linkers used in reversible PEGylation.



(3.28)

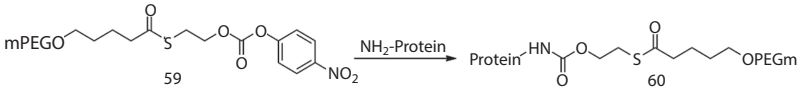
As expected, the rate of release can be modulated by modification of the heteroatom that brings together the polymers and the linker (X in compounds **53** and **54**), by introduction of additional side chains, or by increasing the steric hindrance. Related to mPEG **3**, bicine linkers **53** are particularly interesting and useful structures, due to the well-known advantages that have been described previously for branched mPEG polymers.

Fluorenyl-based reagent **58** has been used for the attachment of mPEG of up to 40 kDa to proteins by means of urethane bond formation (Scheme 3.11). The fluorenyl moiety releases the native unmodified protein at a slow controlled rate by hydrolysis reaction, as is shown in Equation 3.29 [216]. Reagent **58** has been used in the reversible PEGylation of exendine-4 [216], human growth hormone, interferon α2 [217], enkephalin [218], atrial natriuretic peptide [219] and insulin [220].

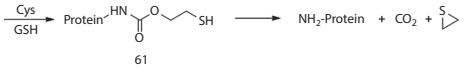


(3.29)

Thioester **59** has recently been introduced for reversible protein PEGylation [221]. Carbamate linkage formation with the target protein affords conjugate **60** (Equation 3.30), which under reductive conditions decomposes to **61**, ultimately giving the free protein (Equation 3.31).

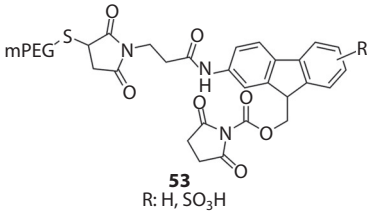


(3.30)



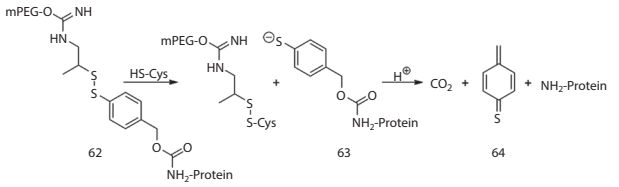
(3.31)

Disulfide bridges can be cleaved under reductive conditions or by sulfide exchange reaction. Thus, this simple concept has successfully been exploited for reversible PEGylation. The chemistry that supports this approach, using conjugate



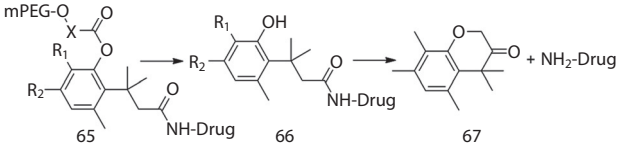
Scheme 3.11 Fluorenyl-based reagent for reversible PEGylation.

62 as an example, is presented in Equation 3.32. Upon reduction of the disulfide bridge of **62** (by a cysteine amino acid, or any other entity containing a -SH group, such as glutatione), intermediate sulfide **63** is obtained. 1,6-Elimination from **63** delivers the unmodified native protein and sulfur product **64** (Equation 3.32) [222,223].



(3.32)

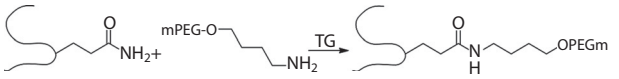
Finally, the reversible PEGylation based in the trimethyl lock lactonization has mainly found application for the controlled release of small prodrugs [224]. The chemistry underlying this process is shown in Equation 3.33. Hydrolysis reaction at a controlled rate of the ester linkage of **65** by endogenous enzymes gives intermediate conjugate **66**, which upon nucleophilic attack of the free -OH group renders lactone **67** and delivers the free native drug (Equation 3.33).



(3.33)

3.6 Enzymatic PEGylation

Conjugation mediated by transglutaminase is nowadays considered as a powerful tool for the site-selective attachment of mPEG to various different proteins. The concept of these conjugations is based on the enzymatic catalyzed acyl transfer reaction between the carboxamide group of glutamine and the -NH_2 group of PEG-amine, which affords stable amide linkage (Equation 3.34). The approach can be used for the PEGylation of native or chimeric proteins, yielding homogeneous conjugates.

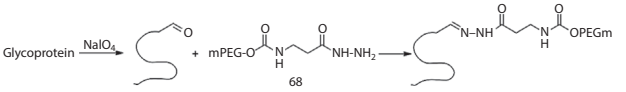


(3.34)

Human granulocyte colony-stimulating factor [225], α-lactalbumin, interleukin-2 [226], salmon calcitonin, IgGH [227], filgrastim [228] and hGH [229], are some examples of proteins that have been PEGylated using transglutaminase. For further insights into this important matter, the reader is referred to the comprehensive article of Veronese and coworkers [226].

3.7 PEGylation of Carbohydrates Residues

Chemical or enzymatic oxidation of carbohydrate residues of glycoproteins generates reactive aldehyde groups that can react with mPEG hydrazines to yield hydrazones, which are usually reduced to the more stable alkyl hydrazide derivatives [230]. To show the concept of these conjugations, the PEGylation with hydrazide **68** is presented in Equation 3.35. Examples of proteins that have been PEGylated following this approach include oxidized ovoalbumin and immunoglobulin G [231].



(3.35)

The related PEGylation of aldehyde groups with PEG-amines is difficult since the -NH_2 groups of proteins show a similar reactivity to the -NH_2 group of PEG-amine, a fact which can result in the unwanted crosslinking of the protein. The *p*Ka of hydrazides is around 3, allowing for selective conjugation at acidic *p*Hs, at which most of the protein amino acids are in the protonated non-nucleophilic form (*p*Ka around 10).

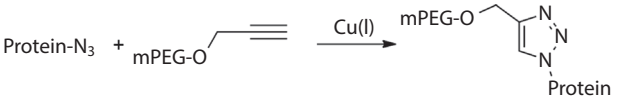
Serine- or threonine-terminated proteins can be oxidized to the corresponding glyoxylyl groups, which can then be reacted with aminooxy mPEG derivatives to achieve site-selective PEGylation. Furthermore, the reactive terminal carbonyl group can be introduced by metal catalyzed transamination [232].

A new two-step process, known as glycoPEGylation, has become increasingly important in the last years for the site-specific bioconjugation of proteins [233]. The sequence for the conjugation consists of a first step of enzymatic glycosylation with *N*-acetylgalatosamine and *N*-acetylgalatosamine transferase at specific threonine and serine residues. In the second step, the protein is PEGylated at the *N*-acetylgalatosamine residue with a sialic acid mPEG derivative using sialyltransferase. Examples of proteins that have been conjugated by this strategy include factor VIIa [234,235], G-CSF, IFN α-2b and GM-CSF [233]. In a related approach, a transpeptidase (sortase A), has recently been employed for the site-selective attachment of mPEG to citokines [236].

3.8 PEGylation by Click Chemistry

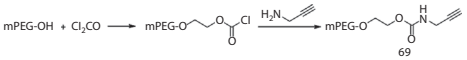
In the last few years PEGylation processes employing "click" reactions have evolved as an innovative strategy for site-selective conjugation [237,238]. "Click chemistry" is defined as a group of reactions that: a) are easy to perform and have wide scope (simple reaction conditions, insensitive to oxygen or water, which can give the desired products from readily available starting materials); b) afford high yields of products (without involving complicated purification steps); and c) give products that are stable under physiological conditions. Although various different systems have been developed for "click" synthesis, the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition (HDC) to form 1,2,3-triazoles, is particularly well suited for site-selective

PEGylation of proteins. Thus, proteins which have been incorporated with azido-containing non-natural amino acids react with mPEG-alkynes affording triazole linkages, as depicted in Equation 3.36 [239].

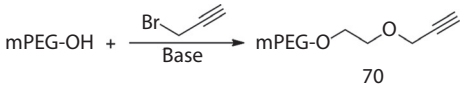


(3.36)

Noteworthily, the 1,2,3 triazole linkage formed upon PEGylation is stable to hydrolysis and this group is usually seen as equivalent to the amide linkage [237]. Another important issue arises from the fact that azide and alkyne functions are unreactive towards most functional groups of natural amino acids, and thus make these processes very site-selective [237]. The -N_3 group that allows the site-selective PEGylations is usually introduced by incorporation of homoazidoalanin into a recombinant protein, among other methodologies [240]. This function serves as attachment site for the covalent conjugation of the protein with the corresponding mPEG-alkynes. Although various mPEG-alkyne derivatives are commercially available, some of them can be obtained following simple synthetic steps, as shown for the preparation of mPEG-propargyl carbamate **69** (Equation 3.37) and mPEG-propargyl ether **70** (Equation 3.38) [241–243].



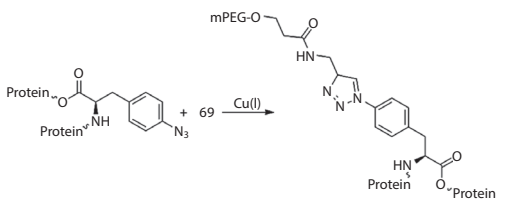
(3.37)



(3.38)

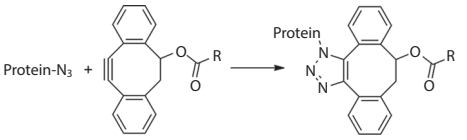
The most common and convenient catalytic system used to trigger the "click" PEGylation includes the use of $CuSO_4$ $5H_2O$ as copper source and sodium ascorbate as mild reducing reagent [239,244]. Other reagents, such as hydrazine [245] and tris(2-carboxyethyl)phosphine [246] can also be used for the reduction of the Cu(II) source to the active Cu(I) species. Even though the methodology complies with all the requisites for a "click" reaction, one major drawback is associated with the use of the copper catalyst, since it is well known that copper compounds can be toxic [247,248]. Moreover, the use of copper salts can lead to protein denaturation. Thus, the required amounts of copper source and reducing reagent, as well as the use of an additive (such as histidine) should all be carefully evaluated, in order to prevent protein denaturation and, eventually, toxicity effects [249].

In a closely related method, the genetically introduced *para*-azidophenylalanine residue was used to achieve the site-specific conjugation of SOD by means of a [3+2] cycloaddition reaction with mPEG-propargyl amine **69** (Equation 3.39) [250].



(3.39)

More recently a new PEGylation method involving the formation of the triazole linkage by [3 + 2] cycloaddition reaction has been developed. Noteworthily, this method does not require the use of copper catalysts. As shown in Equation 3.40, strained cyclooctynes **71** are used as PEGylation reagents, which are aimed at decreasing the activation energy of the "click" reaction [251].



(3.40)

71a: R: NH-PEGm
71b: R: $(CH_2)_3C(O)NH$-PEGm

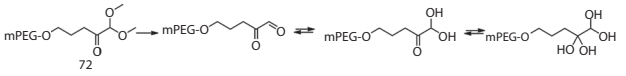
This method has been used in the PEGylation of modified CalB, and it has been demonstrated that the conjugation reactions proceed faster than the analogue processes catalyzed by copper [252].

3.9 Other PEGylations

3.9.1 PEGylation at Arginine

The PEGylation of basic and nucleophilic arginine residues with mPEG phenylglyoxal has been reported some years ago. The usefulness of this methodology was rather limited due to the long reaction times that were required to achieve good yields of conjugation. In addition, the PEGylation with mPEG phenylglyoxal was not selective and conjugation at histidine and lysine residues was also usually observed [253,254].

A more convenient method for the site-selective PEGylation of arginine has only recently been disclosed [255,256]. The process relies on the thermodynamic selectivity obtained upon reaction of the protein with methyl glyoxal analog **72**. In aqueous solution, dimethyl acetal derivative **72** is in equilibrium with the unprotected forms, which are the actual PEGylating reagents (Equation 3.41).

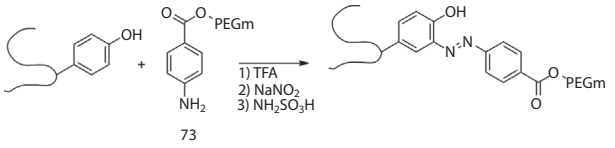


(3.41)

A detailed study of the reaction of methyl glyoxal **72** with *N*-acetyl arginine and *N*-acetyl cysteine has been performed and, importantly, it has been demonstrated that with reagent **72,** PEGylation at lysine residues does not occur [256].

3.9.2 PEGylation at Tirosine

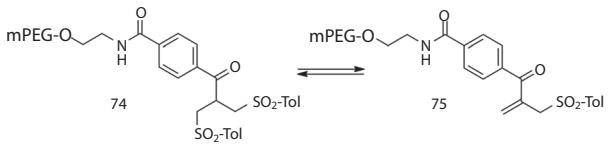
The site-selective conjugation at tyrosine residues of salmon calcitonin using diazonium salt **73** has recently been published (Equation 3.42) [257]. It has been indicated that the PEGylation reaction is reversible, and that the starting materials can be obtained by reaction with suitable reducing reagents.



73 (3.42)

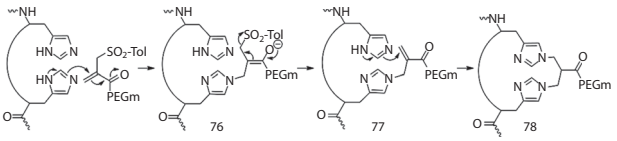
3.9.3 PEGylation at Histidine

Recombinant proteins expressed in several prokaryotic expression systems are often produced with histidine-tags (HisTag), which allows the purification of proteins using affinity chromatography with matrices that contain nickel or other metal ions. Brocchini has nicely shown that these tags can be used for site-selective PEGylation employing new mPEG-sulfone reagents. It is worth mentioning that since the histidine tags are expressed at the N or C terminus of proteins, the biological activity of the protein is not compromised [258]. The concept of the HisTag PEGylation has been exploited for the development of the TheraPeg® technology, which involves a *bis*-alkylation reaction with a double 1,4-Michael-type addition using bis-sulfone **74** as PEGylation reagent. In physiological conditions, monosulfone **75** is formed upon elimination of tolyl-sulfinic acid from mPEG bis-sulfone **74**. Unsaturated sulfone **75** is the actual PEGylating reagent (Equation 3.43).



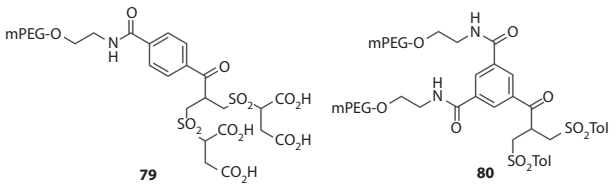
(3.43)

The concept of the conjugation reaction with sulfone **75** is presented in Equation 3.44. The nucleophilic addition of the heterocyclic nitrogen of the aromatic ring to the double bond affords intermediate **76**, which upon elimination of tolyl-sulfinic acid forms α,β-unsaturated ester **77**. A second Michael addition finally gives cyclic *bis*-alkylated product **78** (Equation 3.44).



(3.44)

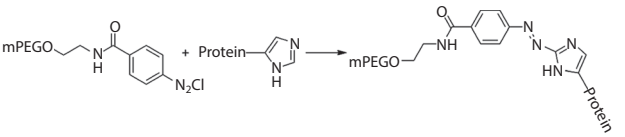
Bis-sulfone **79** and branched *bis*-sulfone **80** have also been prepared and carefully evaluated in HisTag PEGylation (Scheme 3.12) [258].



Scheme 3.12 Reagents for HisTag PEGylation.

Examples of proteins that have been PEGylated by HisTag technology include cytokines (IFN α-2a), antibody fragments (anti-TNF, scFV), endostatin and pro-brain natriuretic peptide.

It has been shown that PEGylation at histidine amino acids can also be accomplished using simple mPEG-diazonium salt **81** (Equation 3.45) [259]. This reagent reacts with the protein (lysozyme) in mild conditions and in short reaction times, affording the mono-PEGylated protein together with a small amount of *bis*-conjugated adduct.



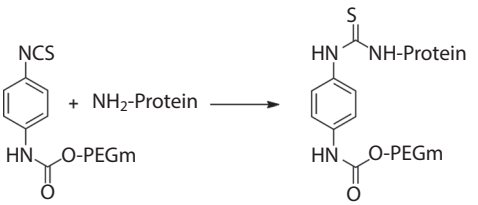
(3.45)

3.9.4 PEGylation at Carboxylic Groups

The challenging site-selective PEGylation of terminal carboxyl groups has been reported by Zalipsky. In this method, mPEG-hydrazide is reacted selectively with the carboxyl group by means of carbodiimide activation at low *p*H. At the *p*H values at which the conjugations are performed (4.5–5.0) all amino groups of the protein are in the protonated non-nucleophilic form and, hence, the crosslinking of the biomolecule does not occur [260].

3.9.5 PEGylation with mPEG Isothiocyanate

PEGylation of amino groups of human serum albumin and human keratinocyte growth factor 2 using mPEG isothiocyanate has been reported [261,262]. The conjugation with this reagent affords hydrolytically stable thiourea linkages by means of a simple and easily obtained PEG derivative (Equation 3.46) [261].



(3.46)

Other methods for PEGylation include the conjugation of non-natural amino acids, such as *p*-acetylphenylalanine with mPEG-oxyamine [263,264], pyrrolysinine and pyrroline-carboxy-lysine with mPEG carbonyl compounds [265], among several other methods [266–268].

3.10 Actual Trends

Optimization of PEGylation processes, analyses of PEGylation reaction mixtures and characterization and activity studies of purified conjugates are usually extremely

time- and cost-consuming tasks. A promising automated process for screening the experimental conditions in the microscale, as well as for characterization of the PEGylation of lysozyme with mPEG-succinimidyl carbonate **2** and mPEG-propionaldehyde **4**, has recently been reported [269].

Solid-phase PEGylation is an increasingly important technology that is expected to gain relevance in the future. In these conjugations the proteins are PEGylated using mPEG reagents that are linked to a solid matrix. The main goal of the methodology is to surpass some of the drawbacks that are commonly found in solution-phase methods. Several approaches have been developed, including site-specific PEGylation of keratinocyte growth factor 1 [270] and the use of heparin-sepharose columns to conjugate recombinant human fibroblast growth factor 2 to PEG-butyraldehyde through reductive alkylation [271]. Other examples include the conjugation of albumin and staphylokinase [272], lipopetides [273], interferon α-2a [274] and hemoglobin, among others [275].

Further innovative PEGylation strategies include: i) C-terminal PEGylation of recombinant proteins expressed as intein-fusion proteins, using hydrazone-forming ligation reactions [276]; ii) PEGylation of latex surfaces beads for immunocamouflage [277], iii) PEGylation of immunonanoparticles [278], and iv) monoPEGylation using an adapted Dock and Lock (DNL) method [279].

3.11 Conclusions

The number of peptide and protein drugs amenable for bioconjugation, as well as new PEG tailor-made reagents and PEGylation strategies, are still increasing nowadays. Actual developments in PEGylation technology tend to tackle the two major difficulties found in bioconjugation, namely: i) to guarantee product homogeneity by improving site-specific approaches, and ii) to diminish the cost of production processes by improving the efficiency of the conjugation reaction, while maintaining batch-to-batch reproducibility.

As was shown earlier, one of the most effective strategies to achieve site-selective conjugation involves PEGylation at scarce cysteine residues located at the surface of the protein.

PEGylation is undoubtedly a diverse and hot field, which is embedded within emerging technologies for the development of improved and new protein therapeutics and other applications. PEGylation takes place at the interface of chemistry, biotechnology and polymer chemistry, as well as pharmaceutical and biomedical sciences. Thus, it is synonymous with inter- and multi-disciplinary research, a key factor for success that is not new but scarce in a situation where finding a common language is sometimes difficult. The combination of these and other factors, such as the ever-growing demand and increasing complexity emerging from the need for more effective therapeutic targets, even today still stimulate the development of research projects aimed at the design, synthesis and evaluation of tailor-made mPEG reagents and technologies. We believe that these efforts will provide, in the near future, further interesting and technologically relevant results.

Acknowledgements

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4

Critical Points and Phase Transitions in Polymeric Matrices for Controlled Drug Release

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Abstract

Polymeric matrices are the most widely employed systems for preparation of controlled release pharmaceutical dosage forms due to their simple and low-cost manufacturing process.

Percolation theory studies the critical concentration of the components of disordered systems. When a component reaches its percolation threshold, it undergoes a geometrical phase transition and starts to extend over the whole sample, having much greater influence on the properties of the dosage form.

This chapter describes the main polymers employed in the manufacturing of pharmaceutical matrix systems. Furthermore, the principal factors affecting drug release from polymeric matrices are analyzed from the point of view of the classical theories as well as the percolation theory.

Keywords: Controlled drug delivery, percolation theory, critical points, cellulose derivatives, polymeric matrices, relative particle size, tablet porosity, biodegradable polyurethane

4.1 Introduction

Polymeric matrix systems are the most widely employed for the formulation of oral prolonged release pharmaceutical dosage forms due to their simple and low-cost manufacturing process. There are three main types of pharmaceutical matrices: inert, hydrophilic and lipidic matrices, being hydrophilic and inert matrices the most employed ones..

Percolation theory studies the critical points of a wide variety of systems and processes. When this theory is applied to pharmaceutical systems, i.e., tablets, pellets, etc., the critical points correspond to the critical concentration of one or more components of the system, including the system porosity. When one component of the system reaches its percolation threshold it experiences a geometrical phase transition and starts to span the whole sample, having stronger influence on the properties of the system, which usually undergo abrupt changes.

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Since the first time that this theory was applied to the pharmaceutical sciences by Leuenberger and coworkers in 1987, many studies have been carried out applying their concepts to the design and characterization of inert and hydrophilic matrices manufactured with the most employed commercial polymers and, more recently, with new biodegradable polymers. These studies have shown that critical points govern the drug release from polymeric matrix systems. Therefore, their knowledge is essential for a correct application of the concept of Quality by Design, established in the directives of the International Conference on Harmonisation (ICH Q8: Pharmaceutical Development).

The purpose of this chapter is to provide a description of the different types of polymeric matrices existing in the market and how the properties of the principal polymers employed in their manufacture affect the drug release. Furthermore, the main concepts of percolation theory are exposed in order to explain the critical points influencing the drug release from matrix systems.

4.2 Matrix Systems

In the last decades, great attention has been paid to the development of oral prolonged release drug delivery systems. These systems are pharmaceutical formulations that release the drug slowly and in such a way that the release rate becomes the limiting step in controlling the arrival of the drug to systemic circulation, so that the drug plasma levels remain more constant with less fluctuation between peaks and valleys than in the case of conventional dosage forms. Therefore the therapeutic concentration of drug is maintained over a longer period of time and the time between doses is increased. This fact improves patient compliance, which is one of the main factors affecting the success of a treatment. Furthermore, toxicity caused by overdose is reduced as well as problems of effectiveness due to valleys in the drug plasma levels. Nevertheless, these systems show some drawbacks such as difficulty of eliminating the drug quickly in case of adverse effects, intra- and inter-individual variability in the drug plasma concentrations due to the dependence on gastric emptying (especially in the case of monolithic systems) and risk of burst effect caused by the breakage of the systems when the dosage form is broken, chewed or crushed [1].

Matrix systems have a high interest in the formulation of prolonged drug delivery devices because of their low cost and ease of manufacture. Matrix systems consist of a drug dispersed in a polymeric surrounding substance. The drug can be dispersed at a molecular level, although it is more usually in the form of solid particles. Despite the fact that the polymer can undergo swelling and/or erosion processes, it must keep the integrity of the system during the drug release process. The progress achieved in the manufacturing of matrix systems depends on the advances of the polymer science and the knowledge of the factors affecting their behavior [2].

Depending on the polymer nature, matrix systems can be classified in three different categories:

- Inert matrix systems;
- Hidrophylic or swellable matrix systems;
- Lipidic matrix systems.

More recently, combined matrix systems prepared with a mixture of an inert and a hydrophilic polymer are being studied.

Depending on the number of particles that constitute the dosage form, matrix systems can also be classified into monolithic systems, such as tablets, or multiparticular systems, such as, for example, pellets. Multiparticular systems offer several therapeutic advantages in comparison with monolithic systems, such as dispersion as individualized units in the gastrointestinal tract, reducing high local drug concentration. This fact leads to a maximization of the drug absorption and a reduction in the peak plasma fluctuation. Moreover, the drug effect is less dependent on the gastric emptying, which reduces intra- and inter-individual variability of the drug plasma concentration [3,4].

4.2.1 Inert Matrices

Inert matrices are manufactured with polymers that form an indigestible and insoluble skeleton after compression. These polymers must accomplish different requirements such as ability to form a porous and non-disintegrable net after compression, insolubility in the gastrointestinal fluids, compatibility with the drug and other excipients, and lack of toxicity [2]. Examples of materials that are used as inert matrix-forming polymers are metacrylate copolymers (Eudragit®RS), ethyl cellulose, dibasic calcium phosphate, polyvinylacetate (PVA), etc.

Drug release from inert matrix systems occurs by diffusion through the pores of the matrix (once the gastrointestinal liquids spread across the porous net by capillarity, causing the dissolution of the drug), including the initial pores and the pores that appear once the drug is dissolved [5]. Equation 4.1 that describes the diffusional drug release kinetics from inert matrices was proposed by Higuchi [6]:

$$Q = S\,[(D\cdot\varepsilon/\tau)\,(2A - \varepsilon\cdot C_s)\,C_s\cdot t]^{1/2} \tag{4.1}$$

Where:

S: Surface of the drug exposed to the dissolution medium;
Q: quantity of drug released from the matrix by unit of exposed surface at time t;
D: diffusion coefficient of the drug in the dissolution medium;
A: amount of drug by unit of matrix volume (concentration in the matrix);
C_s: solubility of the drug in the dissolution medium;
ε: porosity of the matrix;
τ: tortuosity of the matrix.

This model can be referred to as a "classical model." Some years later, Gurny *et al.* described the existence of zero-order release periods in inert matrix tablets prepared with ethylcellulose [7]. These periods were explained by the saturation of the drug in the water-filled pores of the matrix. When the saturation conditions are kept for a significant time period, the dissolution rate becomes slower than the rate of diffusion and therefore becomes the rate-determining step of the drug release kinetics. Potter *et al.* and Caraballo *et al.* also confirmed the existence of these periods in compact and inert matrix tablets, respectively [5,7].

4.2.2 Hydrophilic Matrices

One of the most employed types of controlled drug delivery systems is swellable matrices, which consist of a homogeneous dispersion of drug molecules in one or more hydrophilic excipients, which swell after contact with water, generating a gel or a high viscosity colloid. Among the advantages of these systems are highlighted their low cost and simple manufacture, low risk of dose dumping, flexibility to obtain a desirable drug release profile and a wide variety of excipients with low toxicity that can be employed in their manufacture [8].

Cellulose derivatives are the polymers most widely used in the formulation of hydrophilic matrices, although there are a wide range of polymers that can be employed such as polymers of natural origin, like alginates or chitosan, and polymers of semisynthetic origin, such as modified starches, polyurethanes, etc.

Drug release kinetics from these systems depend on several factors such as rate of polymer swelling, rate of penetration of water through the matrix, rate of dissolution of the drug in the medium, rate of diffusion of the drug through the swelled polymer and erosion of the swelled matrix. Due to the complexity of mechanisms involved in the release process from these systems there are a high number of publications that investigate drug release from hydrophilic matrices [9–11].

The mechanism and kinetics of drug release are influenced by the solubility of the drug and the swelling and erosion properties of the polymer. Some authors indicate that water-soluble drugs are released predominantly by diffusion with a limited contribution from matrix erosion, while water insoluble drugs are released mainly through matrix erosion exhibiting time-independent or zero-order release kinetics [12].

Other factors such as polymer concentration, polymer properties, drug content, drug and excipient relative particle size, and compression pressure have been demonstrated to influence drug release [13].

4.2.3 Lipidic Matrices

Lipidic matrices are constituted by a drug suspended or dissolved in lipidic excipients in which the drug is embedded.

The excipients employed are glycerides, fat acids and alcohols, fat acids and low molecular weight esters and waxes. These excipients are generally of natural origin and physiologically tolerated. The mechanism of drug release depends on the characteristics of the excipient employed. In the case of digestible lipids, the matrix is destroyed by the hydrolysis of the fat components and the erosion is the predominant mechanism. By contrast, in the case of non-erodible excipients, the drug is mainly released by diffusion through the excipient.

4.3 Polymers Employed in the Manufacture of Matrix Systems

There are a wide range of excipients which can be used in forming matrix systems, and among them, polymers constitute the largest group. The following are the most frequently used in inert and hydrophilic matrices (Table 4.1).

Table 4.1 Polymers employed in the manufacture of inert and hydrophilic matrices.

Inert Matrices	Hydrophilic Matrices
Ethylcellulose Polymethacrylates Polyvinyl acetate	Cellulose ethers (HPMC, MC, CMC) Chitosan Gums (Xanthan gum, Guar gum) Polyethyleneoxide Polyurethanes Sodium alginate Starch derivatives

4.3.1 Polymers for Inert Matrices

4.3.1.1 Ethylcellulose

Ethylcellulose is an ethyl ether of cellulose, prepared by ethylation of the alkali cellulose, whose most important property is to be insoluble in water. Different ethylcelluloses are commercially available varying both the ethoxyl content (45.0–49.5%) (Ethocel®, by Dow Chemical Company) and viscosity values (10–100 cP). As a general rule, viscosity increases as the molecular weight (MW) of polymer increases. The lower viscosity grades of this polymer are more compressible and compactible, leading to harder tablets and, consequently, to lower drug dissolution rates [14–16]. This increase in compactibility has been explained based on the fact that the polymers with lower MW show a less ordered structure [14].

4.3.1.2 Polymethacrylates

Polymethacrylates are synthetic cationic and anionic polymers of dimethylaminoethyl methacrylates, methacrylic acid, and methacrylic acid esters in varying ratios. This group includes a variety of copolymers with a molecular weight typically higher than 100,000. Several types are commercially available as the dry powder, aqueous dispersion, or organic solution. For sustained drug release, Eudragit® RL and RS by Evonik are the most representative products. Both are water-insoluble copolymers of ethyl acrylate, methyl methacrylate and a low content of methacrylic acid ester with quaternary ammonium groups. The ammonium groups are present as salts and give rise to pH-independent permeability of the polymers.

Moreover, polymethacrylic acid has been successfully blended with other polymers, both physical and chemically, by copolymerization. For example, physical mixtures with native starches, which are not suitable for delayed drug release, have been reported to be useful as drug delivery systems and to guarantee the insolubility of dosage forms in the acidic medium [17]. In relation to chemical blends, methacrylic monomers are especially indicated for the modification and improvement of natural biopolymers, since they have unique characteristics such as copolymerization ability, stability, and efficacy, which compensate for its high cost [18–21]. Grafting of methacrylic monomer on carbohydrate backbone causes important modifications on the physicochemical properties of the final product such as lower crystallinity and density, higher hydrophobicity

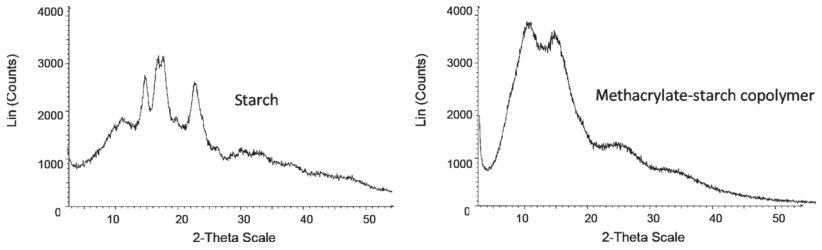


Figure 4.1 X-ray diffractograms of methacrylate-starch copolymer and native starch showing the amorphization of the polysaccharide.

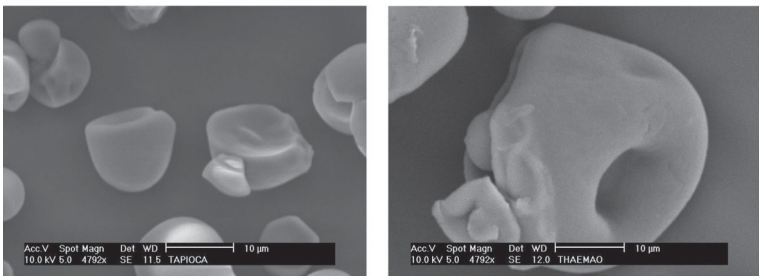


Figure 4.2 Microphotographs corresponding to tapioca starch and methacrylate-starch copolymer showing the increased size of particles.

and an increase in the size of particles [22] [23]. Figures 4.1 and 4.2 show examples of these changes on starch particles.

With respect to technological properties, methacrylic starch copolymerization also improves the compression characteristics of native polysaccharide, increasing the deformation capacity, decreasing the necessary pressure to obtain tablets and improving the friction properties. Nevertheless, the most significant technological improvement can be the reduction of the drug release rate from matrix systems [22]. While standard starch tablets show 100% of drug release at the first hour with a complete disintegration, graft copolymers produce a delayed release and maintain the inert structure of the matrix.

4.3.1.3 Polyvinyl Acetate Mixtures

Polyvinyl acetate (PVAc) is a homopolymer synthesized from vinyl acetate monomer using a free radical polymerization procedure [25]. This water-insoluble polymer is mostly employed blended with other polymers. The most famous association is the physical mixture of PVAc and polyvinyl pirrolidone (PVP), commercially known as Kollidon SR® by the chemical company BASF. Kollidon SR consists of 80% PVAc, 19% of PVP and 1% of sodium lauryl sulfate and silica as stabilizers (BASF). The PVP is added as water-soluble polyamide which forms pores into the matrix tablet allowing drug diffusion. Kollidon SR has been shown to be a suitable pH-independent excipient to form matrix tablets, controlling drug release by both diffusion and erosion mechanisms [24].

4.3.2 Polymers for Hydrophilic Matrices

A variety of polymers are employed as hydrophilic matrix-forming excipients whose characteristics may play a key role and significantly influence the behavior of these devices. Cellulose ethers, especially hydroxypropyl methylcellulose (HPMC), are frequently used as the basis for preparing hydrophilic matrix tablets.

4.3.2.1 Cellulose Ethers

Cellulose is a highly regular polymer of (β-1,4) D-glucose units with a molecular weight of approximately 10^6 Da. This polymer has a rigid and crystalline structure and strong intermolecular hydrogen bonds via hydroxyl groups. Therefore, pure cellulose is not soluble in water. However, cellulose can be modified by the substitution of hydroxyl groups at positions 2, 3, and 6 of the anhydroglucose. Cellulose ethers are produced by incorporating methoxy or hydropropoxy within the glucose chains. Thereby, the crystalline structure of cellulose breaks down and it becomes soluble in water.

4.3.2.1.1 Hydroxypropyl Methylcellulose

Hydroxypropyl methylcellulose (HPMC) or hypromellose is the most frequently used polymer to prepare hydrophillic matrix systems. Some of the reasons for this success are: its non-ionic structure, which may reduce the incompatibilities with other substances; its resistance to enzyme degradation; its stability at a broad pH range (3–11); and its gelification with water. This cellulose derivative, with a MW ranging from 10,000 to 1,500,000, has two different substituents incorporated into the general structure: methoxy (CH_3O) and hydroxypropoxy ($CH_2OCH(OH)CH_3$). HPMC is commercially available with different degrees of substitution, defined as the average number of hydroxyl groups substituted per glucose unit, and viscosity values ranging from 3 to 200,000 cP in a 2% water solution. An example of HPMC 4000 cP is shown in Figure 4.3. Both degree of substitution and viscosity are factors which affect the properties of HPMC, as they determine the formation of the gel layer and affect the rate and mechanism of drug release [25].

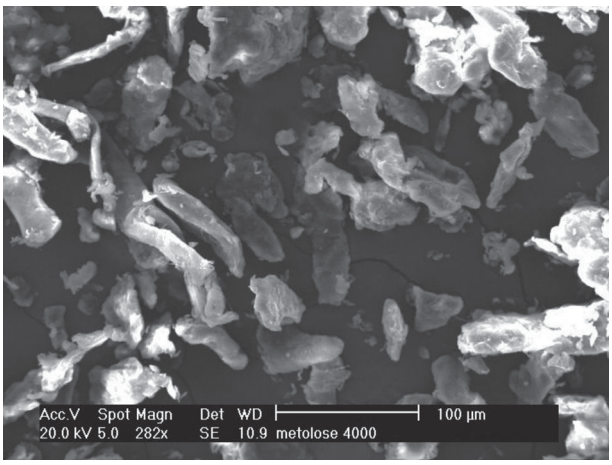


Figure 4.3 Microphotograph showing HPMC 4000 cP particles.

Matrices prepared with HPMC are not significantly affected by the variation of the pH of the dissolution medium. In contact with dissolution fluids, HPMC hydrates slowly, swells and forms a thick gel layer at the tablet surface, which is responsible for controlling the drug release rate [26].

4.3.2.1.2 Methylcellulose

Methylcellulose is a water-soluble cellulose derivative in which approximately 26–33% of the hydroxyl groups are in the form of the methyl ether. The different methylcelluloses show varying degrees of substitution (between 1.4–2.0) and MW (10,000–220,000 Da) [27]. The solubility, as well as other polymer properties, depends on how uniform the methoxyl groups are distributed along the polymeric chain. In matrix tablets, methylcellulose is normally used in combination with HPMC.

4.3.2.1.3 Sodium Carboxymethylcellulose

Sodium carboxymethylcellulose (NaCMC) is an anionic water-soluble polymer prepared by reacting cellulose with sodium monochloroacetate. Typical molecular weight is 90,000–700,000 Da [27]. Various viscosity grades are commercially available, reflecting different degrees of substitution, and consequently, varying aqueous solubility. NaCMC-based matrix tablets control the drug release depending on the pH. Such sensitivity to pH of the dissolution media is attributable to the ionic nature of the polymer. At pH 1 the gel layer formed is rigid, typical of a partially crosslinked hydrogel, with dissolution profiles very similar to HPMC. Drug release from these matrices is driven by diffusion. However, at pH 4.5 and 6.8, NaCMC matrices release the drug faster than HPMC, with a release mechanism mainly governed by polymer relaxation and erosion [28].

Blends of NaCMC and HPMC have been frequently used since the mixture of the two polymers can lead to a combination of the two release mechanisms and nearly zero-order release kinetics could be obtained [28].

NaCMC is incompatible with strongly acidic solutions and xanthan gum, and it forms complex coacervates with gelatin and pectin [27].

4.3.2.2 Chitosan

Chitosan is a copolymer of glucosamine and N-acetylglucosamine, produced from the deacetylation of chitin. Chitosan is commercially available in several types and grades that vary in molecular weight (10,000–1,000,000), in degree of deacetylation and viscosity [27]. This is a widely used pharmaceutical excipient due to its favorable properties, such as enzymatic biodegradability, nontoxicity, low cost and biocompatibility [29]. Indeed, chitosan is an excellent viscosity-enhancing agent in an acidic environment, owing to its high MW, and it acts as a bioadhesive polymer due to its positive charge under dissolution [27].

Moreover, its chemical structure allows the incorporation of new chemical groups, providing versatile materials with specific functionalities and modified physical and biological properties [29]. Several researchers have successfully modified chitosan obtaining new properties such as the observed decrease in the glass transition temperature (T_g) that could be related not only to the more bulky side chains incorporated, but also to the higher water incorporation encountered in the new compounds [29].

4.3.2.3 Gums

Gums are biodegradable and nontoxic polymers with a complex and branched structure. They exhibit high cohesive and adhesive properties and swell in contact with aqueous media.They have been successfully used alone or in combination for preparing sustained drug release systems.

Xanthan gum
Xanthan gum is an anionic high molecular weight polysaccharide produced by fermentation by the microorganism *Xanthomonas campestris.* Each xanthan gum repeat unit contains five sugar residues: two glucoses, two mannoses and one glucuronic acid [27]. This polymer is available in different grades with diverse particle sizes. Matrices containing xanthan gum provide prolonged drug release, being the release rate not affected by pH [30,31]. Moreover, drug release is slightly faster in acidic media owing to more rapid initial surface erosion. Due to its anionic nature, xanthan gum shows incompatibility with cationic surfactants, polymers, or preservatives.

Guar gum
Guar gum is a natural polysaccharide obtained from the ground endosperms of *Cyamopsis tetragonolobus (L.)*, and unlike the previous gum, is non-ionic. It is composed of linear chains of D-galactose and D-mannose, with a ratio between 1:1.4 and 1:2, which may be described chemically as a galactomannan [27]. When guar gum is in contact with water it swells almost immediately and forms a highly viscous, thixotropic sol.

4.3.2.4 Polyethyleneoxide

Polyethyleneoxide (PEO) is a non-ionic homopolymer of ethylene oxide, represented by the formula $(CH_2CH_2O)_n$. This biocompatible polymer shows excellent bioadhesive properties so it is commonly used as sustained-release bioadhesive polymer [32]. PEO is available in different molecular weights under the POLYOX™ (Dow) name. Depending on the molecular weight, different dissolution and water swelling rates, viscoelastic behavior of the swollen gel as well as extent and duration of bioadhesion can be achieved [32]. In general, the higher molecular weight grades provide prolonged drug release form matrix tablets [27].

4.3.2.5 Polyurethanes (PUs)

Polyurethanes (PUs) are among the most commonly selected biomedical polymers [33]. They are a versatile class of polymers with interesting physicochemical properties.

The urethane linkage in biomedical PUs can be formed through a two-step process. The initial step is a reaction involving the end-capping of the macrodiol soft segments (e.g., polyether, polyester, polycarbonate, and polysiloxane) with diisocyanate to form a prepolymer. The second reaction is the coupling of the prepolymer with a chain of low molecular weight, generally a diol or a diamine [34]. The hard segment usually refers to the combination of the chain extender and the diisocyanate components. Due to the chemical incompatibility between the soft and hard segments, the morphology of PUs consists of hard segment aggregations to form domains that are dispersed in a matrix formed by the soft segments [35,36]. This unique morphology is responsible for

the exceptional mechanical properties and biocompatibility of the biomedical PUs. For example, depending on the relative molecular weights and amounts of the hard and soft segments, the PU obtained can be elastomeric or rigid. The mechanical properties of PU can also be tailored by changing the chemical nature of the chain extender. Generally, PUs prepared with aliphatic chain extender are softer than those with aromatic chain extender. Biocompatibility of PUs is also closely related to the chemical nature of the different segments. Early studies by Lyman *et al.* showed that changes in the molecular weight of the polypropylene soft segments affect protein adsorption [37]. Lysine diisocyanate and hexamethylene diisocyanate are preferred over aromatic diisocyanates in the synthesis of biodegradable PUs [38]. Biostability has been, and continues to be, a main research focus of PUs. The challenge to maintain long-term *in-vivo* stability of PUs lies in the fact that biodegradation of PUs is a complex and multifactor-mediated process. Mechanisms responsible for PU biodegradation include hydrolysis, oxidative degradation, metal or cell catalysis, enzymatic degradation, surface cracking, environmental stress cracking, and calcification [38].

Well-defined mechanical properties combined with excellent biocompatibility make PUs attractive for the development of drug delivery systems and medical devices, as well as for tissue engineering [39]. The degradability of polyurethanes is becoming a hot topic in the polymer community due to their biomedical applications [38,40–42]. For example, the synthesis of biocompatible and biodegradable polyurethanes is interesting for the design of short-lived biomedical devices such as controlled drug-delivery systems [43–48].

4.3.2.6 Sodium Alginate

Sodium alginate is a natural anionic polymer typically obtained from brown seaweed. This sodium salt of alginic acid is a linear unbranched polysaccharide which contains varying proportions of β-D-mannuronic acid and α-L-guluronic acid. Various grades of sodium alginate are commercially available with varying viscosity levels. The composition and molecular weight of alginates, pH, temperature, or the presence of metal ions are important, as these factors determine the physical properties of the gel formed in aqueous medium. The sensitivity to pH is in relation to the pKa of its monomers. At pH below the pKa of mannuronic acid (3.38) and guluronic acid (3.65), soluble sodium alginate is converted to insoluble alginic acid, which induces crack formation or lamination of alginate matrix tablets, leading to burst release of drug in a gastric environment. This problem results in loss of controlled drug release and, consequently, potentially limits the use of alginate as a single matrix in tablets for oral drug delivery [49]. It was also noted that alginate particle size, viscosity and concentration affect not only the rate of drug release, but also the release mechanism [50]. Based on its swelling and erosion characteristics, it has been reported that alginate can control drug release for 8–10 h [50,51].

4.3.2.7 Starch Derivatives

While cellulose is the most abundant polysaccharide, starch is the principal plant energy storage. Starch is an abundant, inexpensive, natural biopolymer, with two structures based on α-(D)-glucose, branched amylopectin and largely linear amylose, whose ratio

varies depending on the source. The molecular weight can range between 50 and 500 million Da, depending on the origin and the nature of the starch [27]. From a technological point of view, starches possess poor flow properties, elastic deformation during the tableting process and do not provide a sustained drug release [22]. Therefore, they are often used as disintegrant polymers. Due to the fact that native starches do not swell in cold water, they have been modified with processes such as pregelatinization, using different techniques such as spray-drying, extrusion or drum-drying. Pregelatinized starches have been reported to be useful as prolonged drug release excipients [52,53]. Also, carboxymethyl-substituted high amylose starch exhibited effective sustained delivery properties [54–56]. The formation of a satisfactory sustained-release matrix is strongly influenced by both the proportion and the structural characteristics of amylose and amylopectin, which determine the nature of the resultant matrix structure, gel strength, and viscoelastic properties due to their interactions after hydration. For example, waxy corn starch, with nearly 100% amylopectin, may promote extensive interaction among amylopectin molecules and form a strong and viscous matrix capable of sustaining drug release. In contrast, the essentially linear nature of amylose cannot support the formation of a network structure unless amylose molecules were linked together through extensive crosslinking [52].

4.4 Polymer Properties Affecting Drug Release from Matrix Systems

In-depth knowledge of properties of the polymers employed in the manufacture of matrix systems is always necessary due to the high impact they have on their behavior. The polymer properties showing more influence on the drug release are considered in this section.

4.4.1 Mechanical Properties

Mechanical properties of polymers play an important role in the drug release from matrix tablets. For understanding the role of these properties it is necessary to take into account the concept of glass transition temperature. In contrast to small molecules which have the three typical states, i.e., solid, liquid and gas, polymers decompose instead of boiling and can be glassy, rubbery or viscous liquid. At low temperatures, polymers act as brittle solid or glass because there is not enough energy to permit any chain mobility. As temperature increases, the energy allows some chain movement. The temperature at which the polymer changes from glassy to rubbery state is known as glass transition temperature, T_g, and will be a crucial point to forming stable matrix networks.

In a hydrophilic matrix, the glassy soluble polymer transforms into a rubbery state as the water plasticizes it and reduces the T_g. The glassy/rubbery polymer interface will constitute the swelling (transition) front [9].

In the case of inert matrices, mechanical properties will affect the integrity of the system and drug release. All the polymers mentioned before for inert matrices have an amorphous structure with plastic deformation as dominant densification

mechanism. Therefore, they produce a coherent matrix even under low compression forces. However, these polymers have different T_g. While Kollidon® SR (co-processed PVAc and PVP, ratio 8:2), and Eudragit® RS (ammonium methacrylate copolymer) have lower T_g, 35°C and 60°C, respectively, ethylcellulose exhibits temperatures of about 129–133°C. It has been stated that the degree of elastic deformation of these polymers differs according to their T_g [57]. Moreover, the higher elasticity is in relation with less permanent interparticulate bonding, and lower compressibility and compactibility. Thus, the low T_g of Kollidon comes along with low elasticity and high matrix integrity. Correspondingly, the high T_g of ethylcellulose is associated with high elasticity, but also high stability against temperature and humidity influences. On the other hand, studies performed with these inert polymers have shown drug release rates varying in the same order than matrix integrity, i.e., Eudragit RS > ethyl cellulose > Kollidon SR [57].

In addition, in the case of ethylcellulose, there is a correlation between the viscosity grade and the elastic nature, following the rank order: 100 cp > 45 cp > 20 cp > 10 cp. The dependence of the compressibility and compactibility on viscosity grade seems to be due to the increase in polymer order as a result of increased molecular weight [14].

4.4.2 Particle Size

The effect of the particle size of the polymer on drug release from matrix tablets has been widely studied. This factor is related to the entry of water into the matrix and the formation of a gel layer. Thus, small particles, which are close to one another, produce a coherent gel layer faster, and therefore, a slower drug release rate [58,59]. In the same way, an increase in drug release rate occurs when coarser polymer particles are used due to the fact that higher polymer particles need a longer time for water to penetrate, causing swelling [60]. Moreover, a gel layer with larger pore size has been observed when coarser HPMC particles are used.

However, most authors reported that the effect of particle size seems to disappear for matrices containing high polymer concentrations. Classical theories fail to explain the dependence between the influence of the particle size and the polymer concentration [60]. The explanation provided by the percolation theory will be discussed in Sections 4.6.1.2 and 4.6.2.3 of this chapter.

4.4.3 Viscosity

Viscosity has an important influence on the drug release from hydrophilic matrix tablets, conditioning the passage of water through the gel layer during the swelling process [61]. For largely linear polymers, the viscosity is dependent on the chain length. In aqueous dispersion the viscosity of a polymer depends on its chemical structure, its molecular weight and the interactions with the solvent. Normally, high molecular weight polymers increase the viscosity of the system [59]. Regarding drug release rate, an inverse relationship between release rate and gel viscosity has been shown [62,63]. The more viscous the gel layer formed with the entry of water into the matrix, the greater the resistance to polymer erosion and the slower the release rate of the drug [59].

4.4.4 Molecular Size

The large size of polymers is the reason for their unique properties. For example, their size is responsible for their being non-volatile because they have high attractive forces (primary and secondary) and the energy necessary to volatilize them would be sufficient to degrade them [64]. The molecular size of polymer strongly influences the drug release rates, so that it would be very convenient to know the exact MW of the polymer employed to prepare a pharmaceutical dosage form, especially for prolonged release formulations.

However, the measurement of MW requires complex studies due to its high variability and the large size of these molecules. Therefore, a range of MW is usually employed. In general, an increase of the molecular size decreases the release rates [65–68]. However, some authors have performed studies with polymers with similar MW which showed different release rates, so this property is not the sole determinant of the release rate.

The radius of gyration, Rg, is a parameter directly related to polymer MW. The Rg represents the statistical average of the molecular length [69]. This parameter is used to describe the dimensions of the polymer side chain and increases with the increment of MW. Some studies have shown that Rg influences the drug release process, which is not surprising since Rg and viscosity are related to each other, but cannot alone predict the release rates [59].

4.4.5 Substituent Content

The polymer side chains play a crucial role in hydration rate and the diffusion of the drug in the dissolution medium. The type of substituent incorporated alters the solubility, the gel strength, and the swelling and erosion of the polymer. In the case of substituents with polar groups, a reduction in the crystallinity of the polymer occurs, which is reflected in a decrease in its melting point [59]. This also affects the solubility of the polymer in water. In the case of HPMC, the influence of the substituents has been analyzed, showing that the higher the number of methoxyl groups, the slower the drug release rate [70].

Moreover, an increase in hydrophobicity of the polymer has been observed with the presence of longer side chains [59].

4.5 Percolation Theory

Percolation theory is a statistical theory that studies disordered and chaotic systems. The first works were developed in the Second World War by Flory and Stockmayer to describe how small branching molecules react and form very large macromolecules. This polymerization process may lead to the formation of a very large network of molecules connected by chemical bonds, the key concept of the percolation theory. In the mathematical literature, percolation was introduced by Broadbent and Hammersley in 1957 [71].

Studies of the percolation theory were carried out since 1970 by authors such as Essam and Gwilym. Stauffer carried out detailed studies of these concepts and their application in different fields of science.

However, until 1987 this theory was not introduced in the pharmaceutical field. This step was carried out by Leuenberger and his colleagues at the University of Basel [72–77]. Since that moment, the percolation theory has been applied to an important number of pharmaceutical formulations.

The percolation theory is a multidisciplinary theory which studies and evaluates the distribution of the components of disordered and chaotic systems. The main aim is to study properties, parameters or predicting behaviors near to the percolation threshold [71]. This theory allows the analysis of critical phenomena and has been employed in different fields such as physics, chemistry, ecology, biochemistry and epidemiology. A critical point is defined by an abrupt change in the system properties or by the appearance of new system properties [71].

4.5.1 Basic Concepts

Two important concepts of the theory are "cluster" and "percolation threshold." Percolation theory defines a cluster as a group of neighbor positions occupied by the same component. Two positions are considered neighbors when they share a side of their position in the lattice. An infinite cluster or percolating cluster is defined as a cluster that extends though the system and connects all the sides of the lattice [71].

Figure 4.4 shows a square lattice for two occupation probabilities. The percolation of a component depends on the probability, p, that a randomly selected site will be occupied by this component. The expected number of occupied squares is $p \cdot N$, where N is the total number of squares in the lattice, and the probability that these squares are empty is $(1-p)*N$. The probability p can assume values between 0 and 1.

As Figure 4.4 illustrates, for a low occupation probability (p = 0.2 or 20%), a small number of the squares will be occupied. On the other hand, if the occupation probability is high (p = 0.73), then a large number of squares will be occupied. It is important to realize the existence, in this second case, of a cluster that connects the top and bottom, left and right sides of the lattice. This is called a percolating cluster. In the case of an infinite lattice, mathematicians call it an infinite cluster.

The percolation threshold (p_c) is the concentration of a component at which there is a maximum probability of appearance of an infinite or percolating cluster of this

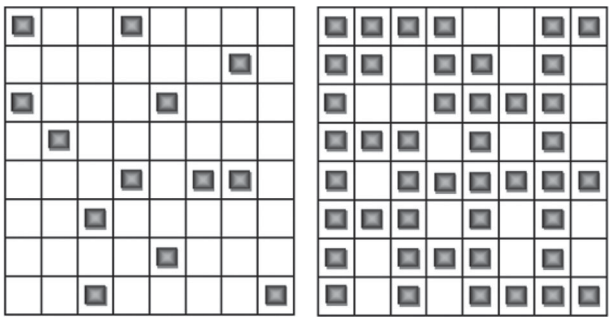


Figure 4.4 Square lattice for two occupation probabilities: (A) Occupation probability (p = 0.2); (B) Occupation probability (p = 0.73).

component. In other words, it is the concentration at which this component starts to percolate the system, acting as the outer phase of an emulsion [71]. At this concentration point, some properties of the system change suddenly.

According to these ideas, a tablet can be described as a heterogeneous binary system formed by a drug and an excipient. As a function of their relative volume ratio, one or both components constitute a percolating cluster formed by particles of the same component that contact each other from one side to the other side of the tablet. The percolation threshold of the drug indicates at which concentration this substance dominates the drug/excipient system. The concept is very similar to the point where a component passes from being the inner to being the outer phase of an emulsion. It is not surprising that the component becoming the "outer phase" or percolating phase will have more influence on the properties of the system [76].

Furthermore, the concentration point at which a component is starting to percolate the system is usually related to a change in the properties of the system, which will now be more affected by this component. This is known as a critical point. Close to the critical point important changes can take place, for example, changes in the release mechanism of the active agent and modification of the tablet structure.

Therefore, in three-dimensional systems of binary mixtures of components A and B, two percolation thresholds (p_c) can be found. As it can be observed in Figure 4.5, starting from low occupation probabilities of component A (therefore, close to 100% of component B in the system), when the concentration of A increases, this component will change its distribution pattern. Component A will pass from being in the form of finite or isolated clusters, to form a percolating cluster. The concentration at which the maximum probability for this geometrical phase transition taking place exists is called the percolation threshold of A (p_{cA}). At the same time, the component B is also forming a percolating cluster of B.

As we move upwards in the plot showed in Figure 4.5, the concentration of component A continues increasing and the concentration of the component B goes down, a critical concentration of B will be found corresponding to the percolation threshold of B (p_{cB}). Below this concentration, component B is expected to be in the form of finite clusters, therefore this component does not percolate the system [78].

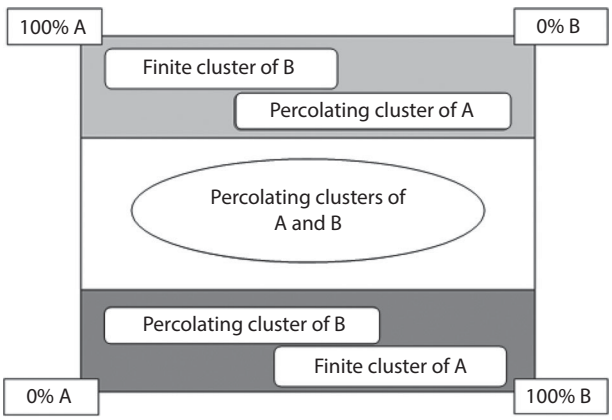


Figure 4.5 Percolation in binary systems.

4.5.2 Fundamental Equation

According to the percolation theory, a system property X at the percolation threshold p_c follows a power law, known as the fundamental equation of percolation theory:

$$X = S\left(p - p_c\right)^q \tag{4.2}$$

where S denotes the scaling factor and q the critical exponent. Equation 4.2 is strictly valid only close to the percolation threshold (±10%). However, in practical cases, Equation 4.2 often showed a much larger range of validity than originally anticipated [79].

The critical exponent q depends on the macroscopic property X. It is interesting to note that the critical exponents which are independent on the type of lattice introduce a kind of universal order in the area of disordered media.

4.5.3 Percolation Models

Different types of percolation models have been developed. They can be divided into random and not random percolation models. In the last ones—correlated percolation, directed percolation, etc.—the occupation probability or the probability of formation of a cluster is conditioned by external factors.

The more important percolation models are the random percolation models. This is especially true when looking to their application in pharmaceutical technology, where for the moment, only random percolation models as site, bond, site-bond or continuum percolation models, have been applied.

Historically, the first one was the bond percolation model, developed in the works of Broadbent and Hammersley. Nevertheless, it was soon observed that the site percolation model provides a more general point of view, so that every situation described by the bond percolation model can also be described by a site percolation model. For this reason, the site percolation model has been the one more exhaustively studied [71].

In site percolation, each lattice site is occupied with probability p and empty with probability *1-p*. A cluster is defined as set of neighboring squares that are occupied (see Figure 4.6).

In bond percolation, the lattice consists of bridges or bonds that can be open or closed (see Figure 4.6). Each bond is open with probability p, otherwise closed with probability *1-p*.

Tablet formation can be imagined as a combination of site and bond percolation phenomena. The formation of a tablet during compression can be described as a site-bond percolation phenomenon. The volume of the matrix is supposed to be spanned by a three-dimensional virtual lattice with lattice spacing of the order of a molecular diameter. This interpretation is more rigorous than an earlier one assuming a lattice spacing of approximately mean particle size, and takes into account a particle size distribution, i.e., a distribution of cluster sizes [76]. After pouring particles/granules to be compacted into the die, the lattice sites are either empty forming pores or occupied by molecules forming clusters.

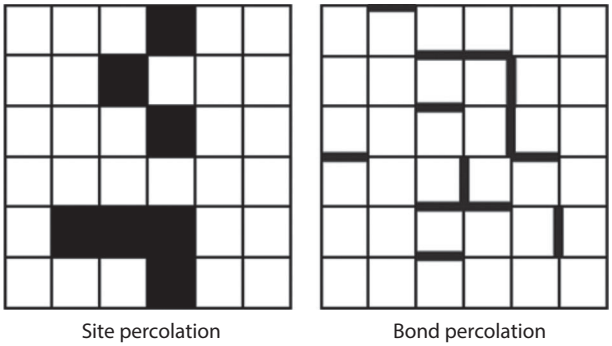


Figure 4.6 Square lattices for site and bond percolation models.

4.5.4 Application of the Percolation Theory to the Design of Controlled Release System

The application of the percolation theory to the design of pharmaceutical dosage forms is a good tool to know more exactly the internal structure of these systems and enables the prediction of the concentrations, which can lead to important changes in the properties of the dosage form (critical points). Making use of this theory, it is possible to reduce time and cost in drug development. It is also possible to produce pharmaceutical forms improving their quality and safety. In addition, the application of this theory allows a deeper knowledge of the formulation structure and behavior, helping to directly design a formulation, knowing that it will fulfill the quality requirements according to the concept of "Quality by Design."

4.6 Critical Points in Matrix Systems

4.6.1 Critical Points in Inert Matrices

There are many factors that affect the drug release from inert matrices such as concentration and solubility of the drug, type of polymer, utilization of polymer blends, particle size of the drug and polymer and hydrophobia of the other excipients that can be employed (fillers, lubricants, binders, etc.).

The concepts of the percolation theory have been employed to explain the influence of the above-mentioned factors in the drug release behavior [77]. Concretely, Leuenberger and Bonny employed for the first time the percolation theory to explain drug release kinetics from inert matrix-controlled release systems [73,74]. To apply the percolation theory to pharmaceutical dosage forms it is necessary to estimate the percolation threshold of the drug and the inert matrix-forming excipient. As it was previously explained, the percolation threshold represents a geometrical phase transition. When a component of the formulation reaches its percolation threshold it starts percolating the system, acting as the external phase of an emulsion. Consequently, when this component is formulated at this or higher concentrations it will have a higher influence on the general behavior of the dosage form [8].

The ideal system for the formulation of a controlled release inert matrix is the bicoherent system which contains percolating clusters of both drug and matrix-forming polymer [5,73,80,81]. Bicoherent systems are possible in solid dosage forms since the percolation thresholds of their components are normally around 30–35% v/v [82]. When the proportion of drug in the system is above its percolation threshold (the drug plus the initial pores percolate the system), the release of the complete drug dose is assured. Otherwise, the drug forms isolated clusters and only the drug fraction contained in the clusters that connect with the surface of the dosage form is released, with part of the drug remaining inside the system, leading to a therapeutic failure. On the other hand, the matrix-forming polymer must also be above its percolation threshold, percolating the matrix. This way the polymer forms a skeleton controlling the drug release and avoiding disintegration of the matrix, which would lead to an abrupt release of the drug.

Besides what has been previously explained, it is advisable not to formulate the system in the closeness of the percolation threshold of its components, since they are areas of great variability that will result in unpredictability in the biopharmaceutical and mechanical behavior. Therefore, the knowledge of the critical points of drugs and polymers, and the influence of the different factors that affect these critical points, lead to more robust formulations and an important decrease in the costs of the optimization process and the time to market [83].

There are numerous studies that have estimated the percolation threshold of different drugs and matrix-forming polymers in inert matrices. The earliest one was carried out by Bonny and Leuenberger [73]. These authors prepared two types of binary matrix tablets employing the water-soluble model drug caffeine with two different polymers: ethyl cellulose (EC) as inert matrix-forming polymer, and hydrogenated castor oil (HCO) as a lipidic matrix-forming excipient. These tablets were prepared with drug loading varying from 10% to 95% (w/w) by direct compression. Matrices were subjected to dissolution testing to measure the amount of drug released versus time. The percolation thresholds of the matrix systems were calculated from these data according to a method developed by the authors [73]. This method employs the β-property, which is a property of the tablet derived from the release profiles and the total porosity of the matrices:

$$\beta = \frac{b}{\sqrt{2 \cdot A - \in C_s}} \tag{4.3}$$

where b is the slope of the Higuchi plot, A the concentration of the dispersed drug in the tablet, ε is the matrix porosity due to the initial tablet porosity and the drug content after leaching, and C_s the solubility of the drug in the dissolution method.

The β property was proposed by Bonny and Leuenberger, knowing that the universal exponent for conductivity and transport properties is $\mu = 2.0$ in three dimensions, and arranging the release parameters in order to have a property that depends linearly on the drug percolation threshold.

Thus, according to the fundamental equation of the percolation theory (Equation 4.2), it can be assumed that:

$$\beta = -c\varepsilon_c + c\varepsilon \tag{4.4}$$

where c represents a constant and ε_c represents the critical porosity-drug percolation threshold. Plotting β versus ε, the drug percolation threshold (ε_c) can be calculated as the point of intersection with the abscissa.

The drug percolation threshold p_{c1} or ε_c, including the drug content and the initial pores for these matrices, ranged between 30–36% (w/w). With respect to the excipient percolation threshold, p_{c2}, differences were found between EC (critical point around 30% w/w) and HCO matrices (6% w/w). The very low percolation threshold of the lipidic matrix-forming excipient was attributed to the different particle sizes of matrix-forming excipient and active substance, being the HCO particle size much smaller than the caffeine particle size. The effect of the particle size of a substance in its percolation threshold will be discussed later.

According to these data, bicoherent systems could be obtained for caffeine loadings between 30% and 70% (w/w) in the case of EC matrices and 45% to 95% (w/w) for the HCO matrices, respectively. So only this narrow range of mixing ratios will provide an almost complete drug release *in vivo* without the risk of disintegration of the matrix.

Melgoza *et al.* carried out another study in which the critical points of binary tablets manufactured with the hydrophobic acrylic matrix-forming polymer Eudragit® RS-PM and the painkiller morphine hydrochloride were estimated [81]. For this purpose matrix tablets with a drug content varying from 10 to 90% (w/w) were prepared. To estimate the polymer percolation threshold, scanning electron mycroscopy (SEM) micrographs of the cross-section of the tablets and the tablet integrity after 20-h release assay were studied. A clear difference in the polymer distribution can be appreciated in the SEM micrographs corresponding to the cross-section of the matrices containing 70 and 80% (w/w) of drug (see Figure 4.7). In Figure 4.7a, it can be observed how in the matrices with a drug loading of 80% w/w, the Eudragit RS-PM particles (dark grey particles in the picture), start to become isolated between the morphine hydrochloride particles. Therefore, the coherent matrix is no longer present and it is foreseeable that the tablet will disintegrate during the dissolution process. With the aim of confirming this result, the tablet integrity after 20-h release assay was studied. The results obtained suggested that the percolation threshold of Eudragit RS-PM in the assayed tablets oscillates between 65 and 80% (w/w) of drug, since tablets containing more than 65% (w/w) of morphine hydrochloride disintegrate after 20 h.

These results are in agreement with those obtained by Melgoza *et al.* and Caraballo *et al.,* who estimated the critical points of matrices prepared with dextromethorphan hydrobromide and naltrexone hydrochloride, respectively, and the same polymer [80,84].

4.6.1.1 Influence of Mechanical Properties of Polymers on Critical Points

In order to determine if the different mechanical behavior of the polymer affects the drug percolation threshold, Eudragit® RS-PM, exhibiting a more rigid behavior, and Ethocel® 100, a plastic excipient, were employed to prepare inert matrices containing KCl as a model drug [85]. The drug percolation threshold of these matrices was estimated employing the method of Bonny and Leuenberger, obtaining 95% confidence intervals of 0.3644 ± 0.0641 and 0.3407 ± 0.0345 of total porosity for matrices

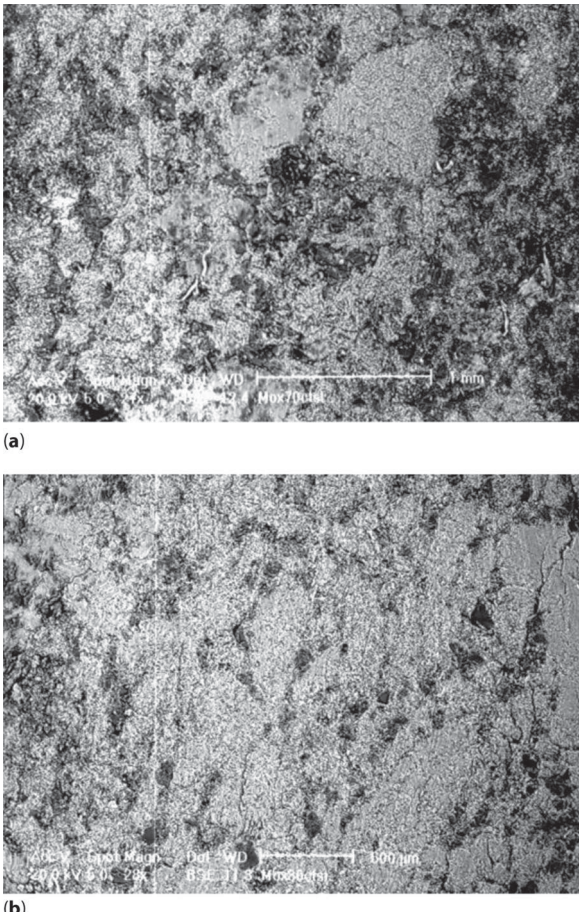


Figure 4.7 SEM micrograph corresponding to the cross-section of the tablets using the BSE detector. (a) Matrices containing 70% of drug. (b) Matrices containing 80% of drug.

containing Eudragit RS-PM and Ethocel 100, respectively [73,74]. Thus, no significant differences were found in the drug percolation threshold in spite of these excipients having very different mechanical behaviors.

4.6.1.2 Influence of Drug and Excipient Particle Size on Critical Points

Different studies have investigated the influence of the particle size of the components of a formulation on their percolation threshold in inert matrices. Initially, the existence of a linear relationship between the drug particle size and the drug percolation threshold was demonstrated, but later studies showed that what really determines the drug percolation threshold is the relative drug particle size, i.e., the ratio between the mean drug and excipient particle size [86,87]. This fact can be explained according to the percolation theory since an increase in the particle size of all the components of a finite system is equivalent to a decrease in the size of the system. Therefore, in a binary

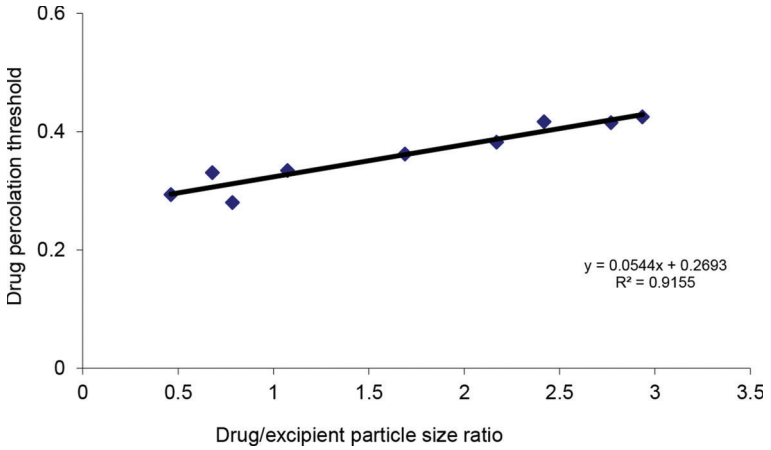


Figure 4.8 Drug percolation threshold (mean±SE) obtained as a function of the drug/excipient particle size ratio employed.

system, the effect of a reduction in the drug particle size is similar to an increase in the excipient particle size. Consequently, an increase in the drug particle size leads to an increase in the drug percolation threshold, while for the excipient particle size the opposite effect is observed. This effect can be explained since a great particle can be considered as a cluster with a 100% density of the same component, which conditions the lower effectiveness of this component to percolate the whole system as an occupation density much lower, of around 50%, is enough to obtain a cluster of the same dimension. This fact results in a higher percolation threshold for this component [71].

The linear relationship between the percolation threshold and the relative particle size can be observed in Figure 4.8 showing drug percolation thresholds as a function of the drug/excipient particle size ratio in inert matrices prepared with different granulometric fractions of KCl and Eudragit® RS-PM. Therefore, drug particles of a bigger size have a low efficiency to percolate the system.

4.6.1.3 Influence of the Utilization of Polymer Blends on Critical Points

In the beginning, the percolation theory was applied to binary systems. However, the majority of pharmaceutical matrix systems are comprised of more than two components, being multicomponent matrix systems frequently employed. That is the reason why studies in ternary and multicomponent matrix systems have been carried out in order to explain the behavior of these matrices [88, 89].

Caraballo *et al.* studied ternary matrix systems containing KCl, polyvinylpyrrolidone crosslinked (PVP-CL) and Eudragit® RS-PM [89]. The *in-vitro* release behavior of these matrices was evaluated on the basis of its dissolution efficiency (E_d), and clear percolation thresholds were not found for the employed components separately. Nevertheless, when the E_d was plotted as a function of the Eudragit RS-PM volume ratio, an abrupt decrease of this parameter could be appreciated at approximately 65% v/v of Eudragit RS-PM (see Figure 4.9). This effect does not correspond to the Eudragit

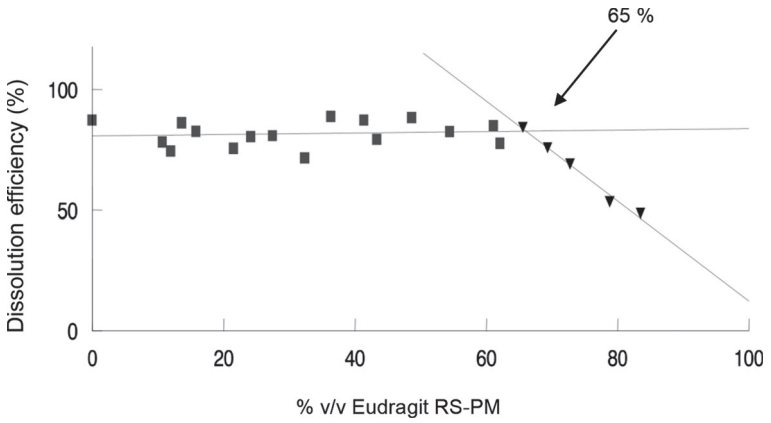


Figure 4.9 Evolution of E_d of the studied tablets as a function of the Eudragit® RS-PM volume fraction (%).

RS-PM percolation threshold, but to the percolation threshold of the hydrophilic substances jointly considered. Therefore, the existence of a combined percolation threshold of the hydrophilic components was demonstrated, showing that this combined percolation threshold can be reached independently on the proportion of each component in the mixture. The only condition is that the sum of the concentration of both components reaches the value corresponding to the combined percolation threshold; i.e., the hydrophilic substances are in a concentration that allows them to percolate the tablet. Consequently, it can be concluded that multicomponent inert matrix systems can be simplified to a binary matrix system on the basis of a discriminatory property, such as the solubility.

4.6.1.4 Influence of Filler on Critical Points

In matrices prepared with drugs showing low water solubility, the presence of a fast dissolution filler has been shown to mask the percolation threshold of the drug, exerting a strong influence in the matrix properties. That was the case of a study carried out by Cifuentes *et al.* in which five lots of matrices containing 30% (w/w) of carbamazepine, different percentages of Ethocel® and from 15.23 to 53.27% v/v of the freely soluble filler lactose were prepared [90]. A clear change in the release behavior was appreciated between batches containing 33.2% (v/v) of lactose (batch FP C3) and 23.9% (v/v) of lactose (batch FP C4) (see Figure 4.10). This change can be attributed to the percolation threshold of lactose, meaning that batches with content up to 23.9% (v/v) lactose do not contain a percolating cluster of the filler, which is encapsulated by the other components of the formulation, resulting in a slower drug release. On the other hand, batches with a content of lactose of 33.2% (v/v) or higher contain a percolating cluster of lactose, so that the filler favors water penetration into the matrix.

Figure 4.10 also shows an unexpected behavior of lot FP C3, as this batch exhibits a very fast initial drug release, faster even than the release rate shown by lots containing a higher content of lactose. This strange behavior can be explained by taking into account that the main components of the matrix—the poorly soluble drug, the

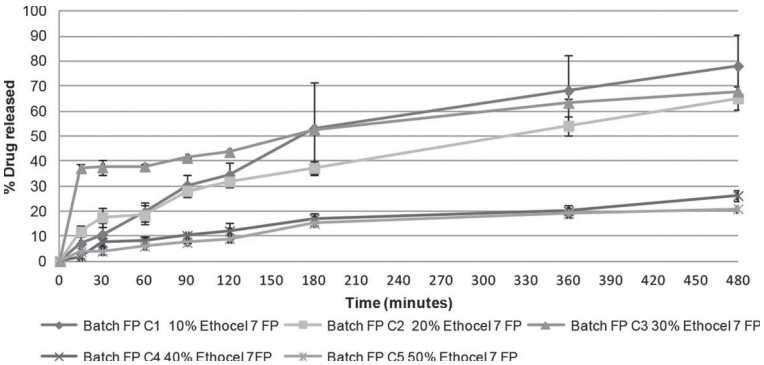


Figure 4.10 Dissolution profiles of batches containing carbamazepine and ETHOCEL® 7 FP.

insoluble polymer and the freely water soluble filler—are above or very close to 30% (v/v); so these three components are in concentrations that allow them to percolate the tablet, leading to a tricoherent system that could be the reason for the anomalous behavior shown by this lot.

4.6.1.5 Influence of Tablet Porosity on Critical Points

In order to investigate the influence of the tablet porosity on the release behavior of inert matrix tablets, Cifuentes *et al.* prepared five batches containing 30% of carbamazepine and 40% of ethylcellulose (Ethocel® 7 FP) as well as 5 batches containing 30% of carbamazepine and 40% of Ethocel® 10 FP with 5 different porosity levels ranging from 0 to 25% [90]. Both types of matrices contained the soluble filler lactose. As it was expected, matrices prepared with both types of polymers and lower initial porosity (0–5% v/v) showed a slower release rate since these matrices were below the percolation threshold of the soluble filler plus the initial porosity.

Considering the effect of the porosity as an individual factor, and according to percolation theory, assuming a random distribution, the percolation of pores through a solid system follows a continuum percolation model, showing a percolation threshold of around 16% [91]. As it can be observed in Figure 4.11, which shows the behavior of the percent of drug released at 1 h, 3 h and 6 h as a function of the initial tablet porosity, the results obtained are in agreement with this hypothesis, given that tablets containing more than 16% of initial porosity show higher percentages of drug released at the three studied time points.

4.6.2 Critical Points in Hydrophilic Matrices

As it has been explained in the previous section, the percolation theory has been extensively employed to describe the behavior of inert matrix systems. More recently, this theory has been applied to the study of swellable matrices being critical points in hydrophilic matrices reported for the first time by Caraballo and Leuenberger in 2004 [92]. An important number of papers dealing with the application of the percolation theory to the study of the release and the water uptake behavior of these systems

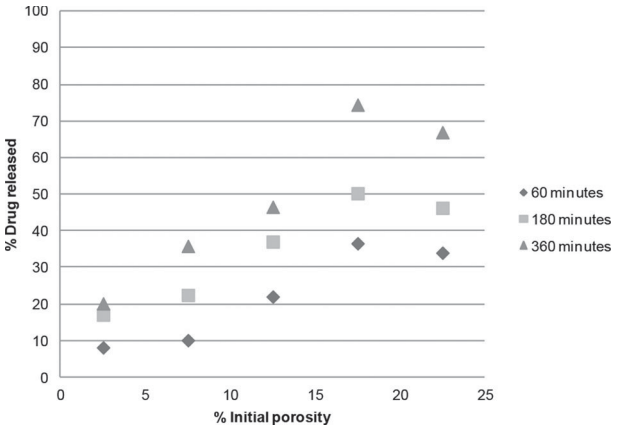


Figure 4.11 Percentage of drug released versus initial porosity of batches containing carbamazepine and ETHOCEL® 7 FP.

have been published since then. Percolation thresholds have been estimated in hydrophilic HPMC matrices, plotting the kinetic parameters (Higuchi's slope 'b'; normalized Higuchi's slope 'b/% (v/v) of HPMC' and relaxation constant of Peppas-Sahlin 'kr') as a function of the volumetric fraction of each component [93–95].

The presence of critical points supposes a discontinuity of the system due to the geometrical phase transition that takes place. This leads to a different distribution of the components of the system. In the case of swellable matrices, critical points can be expected for each one of the components of the formulation. Nevertheless, it has been proved that the polymer critical point plays the most important role in these systems. Above the polymer percolation threshold, this excipient, in contact with the biological fluids, forms a coherent gel layer that acts by controlling the drug release rate. On the contrary, below the polymer percolation threshold, polymer is distributed in isolated clusters which do not produce a continuous gel layer. This leads to the erosion of the polymer and in the majority of the cases to the disintegration of the matrix, with the subsequent abrupt release of the drug in a similar way as a conventional dosage form [60].

With respect to the drug percolation threshold, this critical point shows much less influence on the behavior of hydrophilic matrices when compare to inert matrix tablets. This is attributed to the fact that in swellable matrices the existence of an infinite cluster of drug is not necessary to obtain its complete release. In these systems the polymer swells and enables the water penetration through the whole systems without the need of a percolating cluster of soluble substances. Therefore, little differences have been found between matrices formulated below and above the drug percolation threshold [13, 93, 96].

4.6.2.1 Critical Points in Ternary Polymer Blends

In Section 4.6.1.3 it has been explained the existence of a "combined percolation threshold" in inert matrices, which supposes that this percolation threshold can be reached independently of the proportion of each polymer in the blend with the condition that

the sum of both concentrations reach the value corresponding to the combined percolation threshold. In the case of hydrophilic matrices, ternary tablets containing HPMC and sodium carboxymethyl cellulose (NaCMC) as matrix-forming polymers and KCl as model drug were studied in order to investigate if it is possible to extrapolate the previous conclusion to hydrophilic matrices or contrarily, if the polymers behave in an independent way, being necessary to reach the critical concentration of one of the polymers to obtain the continuous gel layer that controls the drug release [97].

In this study, the percolation threshold of HPMC and NaCMC binary matrices were estimated analyzing the water uptake and the release behavior of the matrices. The HPMC percolation threshold was situated between 29 and 41% (v/v) HPMC for the binary KCl-HPMC matrices, while the excipient percolation threshold for the binary KCl-NaCMC hydrophilic matrices was found between 39 and 54% (v/v) NaCMC. As it can be appreciated, there is a narrow range of overlapping concentrations for the polymers percolation threshold. Thus it suggests that there is a small possibility that these two polymers can show a combined percolation threshold. This hypothesis is confirmed since considerable differences in the Higuchi's slope values (1.416 and 0.49, respectively) were found in two batches of matrices prepared employing very similar volume percentages of both polymers.

On the other hand, an independent behavior of the two polymers has not been confirmed since matrices containing similar HPMC concentrations show important variations in the value of the Higuchi's slope when the concentration of NaCMC varies.

This study concluded that the behavior of the polymers is intermediate between the two models proposed, i.e., there is a collaboration between HPMC and NaCMC to form the gel layer that acts by controlling the drug release rate, although the effect of these polymers is not additive.

Contreras *et al.* also carried out a study in ternary matrices employing the same polymers but using theophylline, a model drug with intermediate water solubility, instead of the freely water-soluble KCl [98]. Percolation thresholds of binary theophylline/HPMC and theophylline/NaCMC hydrophilic matrices have been estimated between 21.5 and 31.3% (v/v) of HPMC and between 39 and 54% (v/v) of NaCMC, respectively. It has been demonstrated that these two polymers do not show a combined percolation threshold for the whole concentrations range. It is necessary that the concentration of both excipients is above a minimum value (approximately between 10 and 20% v/v) to establish an effective collaboration between the polymers [98].

4.6.2.2 Influence of Drug Solubility in Critical Points

As it was previously stated, the mechanism of release of water-soluble drugs is predominantly controlled by diffusion, while the release of poorly soluble drugs tends to be controlled by the erosion of the matrix. In order to study the influence of this factor on the polymer percolation threshold of swellable matrices, Fuertes *et al.* carried out an investigation preparing matrices containing HPMC K4M as hydrophilic matrix-forming polymer and acetaminophen, theophiline and ranitidine·HCl as drugs [99]. The polymer percolation threshold of the different matrices was estimated based on their drug release and water uptake behavior.

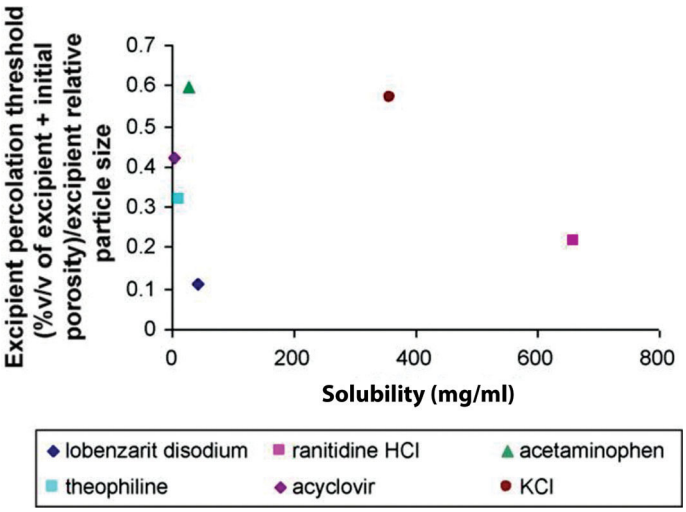


Figure 4.12 Excipient percolation thresholds (expressed as %v/v HPMC + initial porosity)/ HPMC relative particle size versus the drug solubility in matrices containing acyclovir, theophiline, ranitidine HCl, acetaminophen, lobenzarit disodium and KCl.

Since there is a linear dependence of the percolation threshold of one component on its relative particle size, with the aim to correct the influence of the different relative particle size, the values of the excipient percolation threshold were divided by the excipient relative particle size [87]. The obtained results have been plotted against the solubility of the drugs studied in this work: acetaminophen, theophylline and ranitidine·HCl. Moreover, data corresponding to hydrophilic matrices studied in previous works and containing different drugs—KCl, lobenzarit disodium and acyclovir—were also included, as it can be observed in Figure 4.12 [93, 94,96].

As this figure shows, the obtained percolation thresholds are not related to the drug solubility, even making the correction corresponding to the particle size. Different corrected percolation thresholds have been obtained for drugs having very similar water solubility.

These results were confirmed by a study carried out by Gonçalves-Araújo *et al.* in multicomponent HPMC hydrophilic matrices containing verapamil·HCl and carbamazepine [100].

These data suggest the robustness of HPMC hydrophilic matrices and the usefulness of the percolation threshold as a preformulation parameter in order to rationalize the formulation of hydrophilic matrix systems.

4.6.2.3 Influence of Relative Particle Size on Critical Points

As it as been previously explained for inert matrices, the percolation threshold shows a linear dependency on particle size that was found by Caraballo *et al.*, being the factor responsible for the change of the percolation threshold the relative particle size of the studied component [86, 87].

Miranda *et al.* carried out a later study with the aim to investigate if this linear dependence could also apply for hydrophilic matrices [13]. For this purpose, matrix tablets

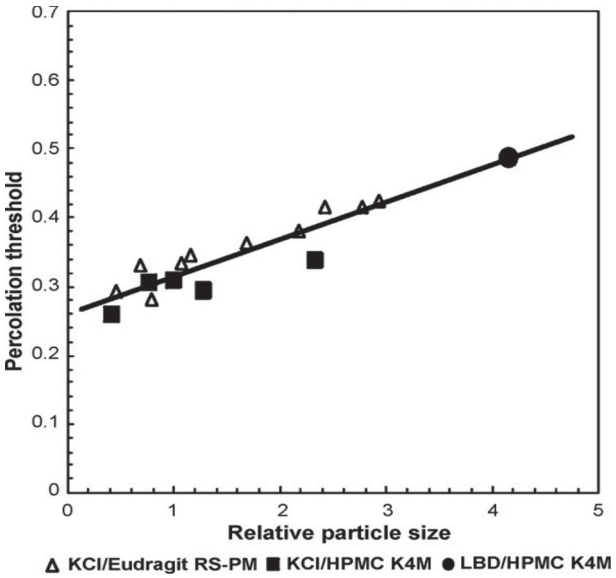


Figure 4.13 Drug percolation thresholds versus the drug relative particle size in inert matrices (KCl/Eudragit RS-PM) and the excipient percolation thresholds versus the excipient relative particle size in hydrophilic matrices (KCl/HPMC K4M and LBD/HPMC K4M).

containing KCl and HPMC K4M as matrix-forming polymer were prepared employing six different excipient/drug particle size ratios (ranging from 0.42 to 4.16). The drug load varied from 20 to 90% (w/w). The polymer percolation threshold was calculated by plotting "b/% (v/v) of HPMC," the normalized Higuchi's slope, versus the HPMC volumetric fraction. The excipient percolation threshold were plotted as a function of the excipient/drug particle size ratio of the matrices but poor correlation parameters were found, so the excipient percolation threshold was calculated adding the initial porosity to the excipient volumetric fraction, and in this case a linear relationship was found. This result suggests that the initial porosity contributes to establish the gel barrier responsible for the release control.

The linear trend was also confirmed for matrices prepared with LBD and HPMC K4M, suggesting that the linear relationship is independent of the drug included in the matrix [96]. Moreover, the same regression line was also found to be valid for systems as different as inert and hydrophilic matrices. Figure 4.13 shows the common regression line obtained for the inert KCl/Eudragit RS-PM and swellable KCl/HPMC K4M and lobenzarit disodium/HPMC K4M matrices.

Therefore, also in the case of hydrophilic matrices, lower percolation thresholds are found for smaller relative particle size of a component due to its higher percolation efficiency.

4.6.2.4 Influence of the Tablet Initial Porosity

Although the influence of the initial porosity has been widely studied in inert matrices, and that it is clear that the pores facilitate the water uptake and the drug release, and that

the initial porosity has to be added to the porosity caused by the dissolution of soluble substances (the drug percolation threshold is expressed as total porosity), the influence of the initial porosity in hydrophilic matrices is not as simple [5,73,74]. As it was explained in the previous section, it has been proposed that the initial porosity contributes to establish the gel barrier, but this supposition was not experimentally confirmed [13]. For that reason, Aguilar-de-Leyva *et al.* carried out the first experimental study of the influence of the initial porosity of the matrices on the polymer percolation threshold, preparing multicomponent carbamazepine formulations and employing HPMC as hydrophilic matrix-forming polymer, MCC and lactose as fillers, and a lubricant mixture. Every formulation was compressed employing three different pressure levels in order to obtain three different levels of initial porosity (mean porosity values: 7.9, 16.0 and 27.3% v/v) [101].

The release behavior of these matrices showed a common critical point of 13–15% v/v of HPMC even though these formulations have three different levels of initial porosity.

Previous works have explained the low values obtained for the polymer percolation threshold in hydrophilic matrices based on the contribution of the initial porosity of the tablet in order to form the gel layer that controls the drug release [13,94,95]. The results obtained by Aguilar-de-Leyva *et al.* do not contradict this hypothesis, since the porosity values of the previous studies correspond to the lower level (5–10%), and this study proposes that it could be an involvement of the initial porosity. Nevertheless, this new study concluded that the contribution of initial porosity to establish the gel layer would be restricted to a low range of tablet porosity [101]. Above this range, an increase in the tablet initial porosity does not affect the polymer percolation threshold.

4.6.2.5 Influence of Polymer Viscosity

As it has been explained in a previous section, it is clear that polymer viscosity influences drug release since an increase in polymer viscosity leads to a greater resistance to polymer erosion and to a slower water uptake and release rate of the drug. Nevertheless, it was unknown if a change in the polymer viscosity also changed the critical concentration of the polymer [101]. In order to study this issue, Aguilar-de-Leyva *et al.* prepared multicomponent carbamazepine formulations employing two different viscosity grades of HPMC as hydrophilic matrix-forming polymer, and carried out the estimation of the critical points of the polymer. The authors found no influence of the two different viscosity grades of HPMC employed in their critical points. This result is in agreement with the approach proposed by the percolation theory, since the critical concentration of a polymer corresponds to a change in the release behavior of the dosage form due to a phase transition of the polymer that passes from being isolated to percolating the matrix. As the distribution of the solid polymer particles inside the matrix is not dependent on the polymer viscosity level, the polymer percolation threshold was expected to remain at the same concentration. As a consequence, the results of this study support the robustness of the percolation threshold and its use as a preformulation parameter.

4.6.3 Critical Points in Multiparticular Matrix Systems

The percolation theory has been widely applied to the study of monolithic matrix systems. However, it was not applied to the study of multiparticular matrix system until the study

published by Aguilar-de-Leyva *et al.* in 2011, employing matrix pellets [102]. Pellets offer some technological advantages compared to monolithic systems such as spherical shape, narrow particle size distribution and low friability. These characteristics are responsible for their very good flow and facilitate their coating or inclusion into hard gelatin capsules or even their compression into tablets [103]. Furthermore, it is possible to combine non-compatible drugs or different drug release profiles in the same formulation [104,105].

Aguilar-de-Leyva *et al.* studied the release profiles of thirteen batches of pellets prepared by extrusion-spheronization process containing varying proportions of clozapine/microcrystalline cellulose (MCC)/hydroxypropylmethyl cellulose (HPMC) and different clozapine particle size fractions [102].

The results of this study show that the distance to the HPMC percolation threshold has an effect on the drug release rate, and as explained in previous sections, this behavior is typical of hydrophilic matrices. Nevertheless, in the studied multiparticulate matrix systems, the drug percolation threshold also exerts an important influence on the release behavior. This is a typical behavior of inert matrices. Therefore, an intermediate behavior between inert and hydrophilic matrices was found for the studied MCC/HPMC multiparticulate matrices.

On the other hand, a clear effect of the drug particle size in the release rate has been appreciated in lots formulated in the vicinity of the drug percolation threshold, being this effect less evident when the distance to the drug percolation threshold increases. This provides a rational explanation to the "gosh" effect of the particle size, previously mentioned by several authors.

Although more research is needed in this field, the percolation theory is providing a science-based explanation of the behavior of matrix systems including inert and hydrophilic matrices in the form of monolithic or multiparticulate systems.

4.6.4 Critical Points in Matrix Tablets Prepared by Ultrasound-Assisted Compression

There are a considerable number of papers dealing with ultrasound-assisted powder compression [106–110].

Caraballo *et al.* applied the concepts of percolation theory to the study of tablets containing KCl as model drug with a content ranging from 10 to 90% w/w and Eudragit® RS-PM (as inert matrix forming polymer), comparing ultrasound-assisted compression and compression in a traditional eccentric machine [91]. The results obtained pointed out that the matrix-forming thermoplastic polymer employed suffered a severe decrease of approximately 50% in its percolation threshold in comparison with the matrices prepared by the conventional compression method, and that a better control in the drug release is obtained.

The reason for the decrease in the polymer percolation threshold was suggested to be that the polymer experiences a sintering phenomenon that results in an almost continuum medium inside the US tablets. The drug particles are practically surrounded by the inert polymer, which diminished the contact with the dissolution medium and therefore slows down the release rate. So, in the case in which a component experiences a thermoplastic deformation, the continuum percolation model can be employed to explain the changes in the system with respect to a tablet obtained by a traditional

compression. The continuum percolation model considers that the component is not distributed into discrete lattice sites. By contrast, this model considers a continuum distribution function of the components. This way the existence of a regular lattice underlying the system is not needed.

As a consequence of the decrease in the polymer percolation threshold, a lower amount of excipient is needed for the manufacture of controlled release tablets by US compression, thus resulting in an important advantage for the pharmaceutical industry that can prepare controlled release matrices containing high drug doses with a low increase in the weight of the final system. As an example of this issue, Aguilar-de-Leyva *et al.* have designed a controlled release hydrophilic matrix tablet formulation prepared with the orphan drug deferiprone that contains only 15% of excipients [111]. This matrix system includes a dose of 750 mg of API—intended for twice a day administration—with a similar weight (ca. 900 mg) to the conventional tablets existing in the market, which contain 500 mg API and have to be administered three times a day.

4.7 Case-Study: Characterization of a New Biodegradable Polyurethane PU (TEG-HMDI) as Matrix-Forming Excipient for Controlled Drug Delivery

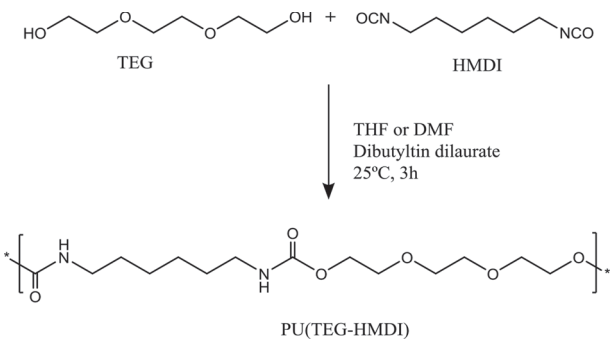
Biopolymers are an essential element in improving human health and quality of life. The wide spectrum of physical, mechanical, and chemical properties provided by polymers has increased the extensive research, development, and applications of polymeric biomaterials. The significance of polymers as biomaterials is reflected in the market of medical polymers, estimated to be approximately $1 billion. Many of these polymers were initially developed as plastics, elastomers, and fibers for nonmedical industrial applications. However, they were later developed as biomedical-specific materials. Currently, with rapid growth in modern biology and interdisciplinary collaborative research, polymeric biomaterials are being widely used in pharmaceutical technology with excellent biocompatibility [33].

The ability of a new biodegradable polymer, polyurethane triethylene glycol 1,6-hexamethylene diisocyanate PU(TEG-HMDI), to act as matrix-forming polymer for controlled release tablets has been studied and its percolation threshold in a matrix system has been estimated [112]. The hydrophilic polyurethane PU(TEG-HMDI) was successfully synthesized by reaction of 1,6-hexamethylene diisocyanate (HMDI) and triethylene glycol (TEG) (Scheme 4.1). The diol monomer (TEG) was chosen to enhance the hydrophilic nature and swelling properties of the new material. These properties contribute to the degradability of the polyurethane.

Rheological studies, as well as drug release studies, were carried out to analyze the properties of the new polymer as matrix-forming excipient.

4.7.1 Rheological Studies

Table 4.2 shows the results of the rheological studies. These results predict that the polymer has adequate flow and compressibility properties, making it a good candidate



Scheme 4.1 Synthesis of the PU(TEG-HMDI).

for direct compression processes, which is an important advantage for preparation of solid dosage forms.

4.7.2 Preparation of Matrix Tablets

Nine-mm diameter binary tablets have been prepared by direct compression in an eccentric tabletting machine (Bonals A-300; Barcelona, Spain). The tablets had a medium weight of 250 mg and contained 10%, 20%, and 30% of excipient PU(TEG-HMDI) and theophylline as model drug.

4.7.3 Drug Release Studies

Figure 4.14 shows the dissolution profiles of the binary tablets prepared. It can be observed that theophylline is released more slowly from tablets containing a higher amount of polymer. For example, in tablets containing 10% of polymer, almost 90% of theophylline is released at 8 hours. However, the percentage of drug released from batches containing 20% and 30% of polymer is lower than 60% after 8 hours of assay [112].

Drug release data have been analyzed according to different kinetic models: Higuchi, Korsmeyer and Peppas & Sahlin [6,113,114]. Table 4.3 shows the results obtained for these models.

As Table 4.3 shows, the obtained time exponents are close to 0.5 for the Korsmeyer equation, indicating that the drug release predominantly follows the mechanism of diffusion, with a low contribution of the erosion mechanism [113].

The results corresponding to the Peppas & Sahlin equation (Table 4.3) are also in agreement with the previous conclusion, being that the values of the diffusion constant k_d are more than 100 times higher than relaxation constant k_r [114].

4.7.4 Estimation of Excipient Percolation Threshold

As explained in previous sections, the percolation threshold corresponds to the concentration of a component for which there is a maximum probability of appearance of an infinite or percolating cluster of this component.

Table 4.2 Bulk and tapped densities, Hausner index, Carr's index, Sponginess index, Rest angle and Flowability of drugs and excipient.

Sample	Bulk Density[a] (g/ml)	Tappped Density[a] (g/ml)	Hausner Index[a]	Carr Index[a] (%)	Sponginess Index[a]	Flowability[a] (g/s)	Rest Angle (°)
PU(TEG-HMDI)	0.099 ± 0.002	0.123 ± 0.003	1.234 ± 0.008	18.94 ± 0.547	1.897 ± 0.061	0.000	42.1 ± 1.9
Theophylline anhydrous	0.330 ± 0.011	0.547 ± 0.005	1.657 ± 0.075	39.559 ± 2.72	1.199 ± 0.125	0.000	50.6 ± 3.8

[a]Mean value ± standard deviation (three replicates).

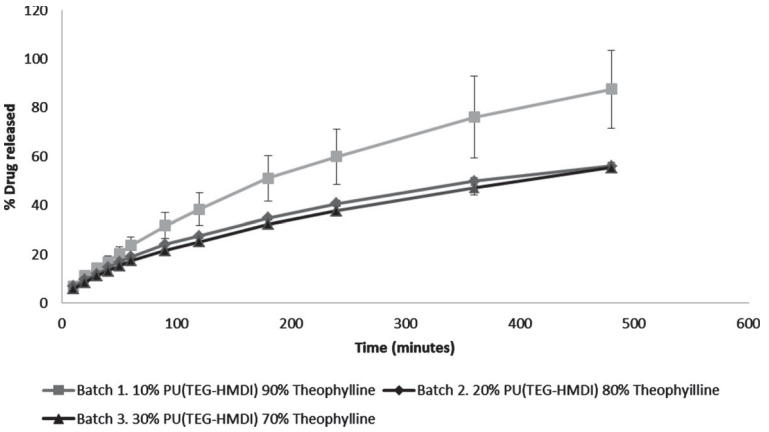


Figure 4.14 Dissolution profiles for batches containing 10, 20, and 30% w/w of PU(TEG-HMDI).

As can be observed in Figure 4.15, the release rate underwent a sudden decrease when the polymer concentration passed from 10% w/w to 20% w/w of polymer. This fact indicates that the batch containing 10% of polymer is below the polymer percolation threshold [93,97,115].

Below the percolation threshold, the excipient does not percolate the system and the drug release is not as efficiently controlled by the gel layer. In this situation, the gel layer initially shows important "holes" which allow a fast water uptake, leading to a quicker release process. By contrast, above the excipient percolation threshold (lots containing 20 and 30% of polymer) a percolating cluster of this component exists and a coherent gel layer is formed from the first moment, which is able to control the hydration and drug release rate.

The new biodegradable polymer PU(TEG-HMDI) shows very good compactibility, allowing it to obtain matrix tablets with a high crushing strength [112]. Moreover, this polymer shows a high ability to control the drug release using very low concentrations.

4.8 Conclusions and Future Perspectives

Polymeric excipients are showing amazing growth in the field of pharmaceutical technology. In this chapter, the behavior and characteristics of the main polymers employed for the preparation of matrix systems that constitute the most popular type of prolonged drug delivery systems have been studied, from both the point of view of "classical" theories, as well as from the new perspective offered by a theory coming from Statistical Physics and named the percolation theory.

On the other hand, in 2004 the U.S. Food and Drug Administration published a directive introducing the concepts of "Quality by Design" (QbD). This initiative encourages a science-based knowledge of the pharmaceutical formulations in order to be able

Table 4.3 Kinetic parameters.

	Higuchi		**Korsmeyer**			**Peppas & Sahlin**		
Batch	$b^a(min^{-0.5})$	r^{2b}	$k_c(min)$	n^d	r^{2b}	$k_e(min^{-0.5})$	$k_f(min^{-1})$	r^{2b}
Batch1 PU(TEG-HMDI)10%	0.044	0.929	0.015	0.669	0.972	0.048	0.00016	0.929
Batch2 PU(TEG-HMDI)20%	0.027	0.997	0.018	0.562	0.996	0.0297	0.00011	0.997
Batch3 PU(TEG-HMDI)30%	0.026	0.993	0.015	0.584	0.994	0.024	0.0001	0.993

[a]Higuchi's slope. [b]Determination coefficient. [c]Korsmeyer kinetic constant. [d]Diffusional exponent. [e]Diffusional constant of Peppas&Sahlin model. [f]Relaxational constant of Peppas & Sahlin model.

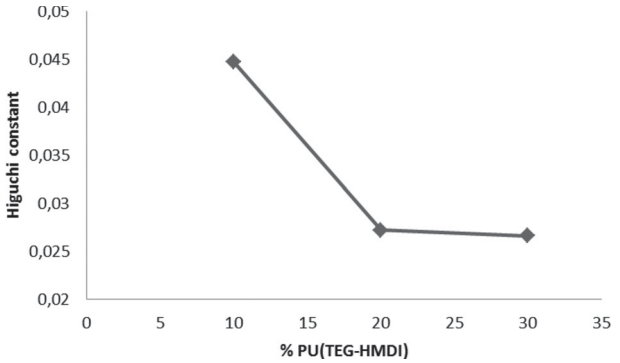


Figure 4.15 Percolation threshold of the PU(TEG-HMDI).

to "design the quality" instead of assaying whether our dosage form meets the required specifications.

One of the main concepts introduced by QbD is the "Design Space," defined as the multidimensional combination and interaction of input variables and process parameters that have been demonstrated to provide assurance of quality.

The concepts of QbD and Design Space are already included in the ICH directives, concretely in the ICH Q8: Pharmaceutical development. Therefore, the regulatory agencies are already asking for the pharmaceutical industry to fulfill these directives.

As explained before, the percolation threshold corresponds to a critical phase transition that supposes an important change in the properties of the system. Therefore, the critical points associated with the percolation thresholds are natural limits of the Design Space. This is an important reason why much more research is expected in this area in the near future.

Furthermore, the explanation based on the percolation approach is more complete than that provided by the "classical theories." Thus it supposes an advance in the concept of "Quality by Design" of the pharmaceutical formulations. Therefore the knowledge of the critical points of the pharmaceutical formulations is essential for medicine manufacturing in the current regulatory environment.

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5

Polymeric Systems in Quick Dissolving Novel Films

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Abstract

Pharmaceutical quick dissolving films are an attractive dosage form for administration of drugs because they dissolve or deaggregate spontaneously in the body cavity, resulting in a solution or suspension without water. These films mainly take advantage of the oral cavity's extremely permeable tissues, which for many years have been a site of absorption for delivery of drugs to the circulation (oral transmucosal delivery, OTD), and for native delivery to the adjacent tissues (oral mucosal delivery, OMD). The fundamental science and up-to-date analysis concerning the oral cavity and oral membrane drug delivery has been reported extensively in the literature.

In this chapter, a systematic approach to outlining the polymers and their possible blends with other excipients has been explored for developing quick dissolving novel films, with special emphasis on their characterization procedures.

Keywords: Quick dissolving films, wafers, polymeric blends, plasticizers, characterization, morphological study, dosage forms, testing, pharmaceutical science, oral delivery, buccal delivery

5.1 Introduction

In order to achieve optimal drug therapy, it is necessary for patients to receive the correct medication, at the right dosage, and at the most convenient dosing intervals [1]. Recently there has been a tremendous need for developing newer dosage forms to improve the efficacy of drugs currently in the market. Due to the low cost associated with both money and time, pharmaceutical companies are reconsidering delivery strategies to improve the usefulness of drugs that have already been approved rather than developing a single new chemical entity. Many pharmaceutical companies have headed their research activity in the direction of reformulating existing drugs into new dosage forms [2]. However, despite the tremendous advances in drug delivery, the oral route remains a well-liked route for the administration of therapeutic agents as a result of low price, simple administration and high level of patient compliance. However, important barriers are obligatory on the

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peroral administration of medicine, like hepatic first-pass metabolism and drug degradation inside the channel (GI) tract, prohibiting the oral administration of certain categories of medicine, particularly biologics (e.g., peptides and proteins). Consequently, different assimilatory mucosae are being thought about as potential sites for drug administration, together with the membrane linings of the nasal, rectal, vaginal, ocular, and oral cavities. The transmucosal routes of drug delivery provide distinct blessings over peroral administration for general drug delivery like the doable bypass of the primary pass impact and turning away of presystemic elimination inside the GI tract. Among these, delivery of medication to the mouth has attracted explicit attention thanks to its potential for top patient compliance and distinctive physiological options. Within the oral mucosal cavity, the delivery of drugs is classified into two categories: (i) local delivery and (ii) systemic delivery either via the buccal or sublingual mucosa [3].

5.1.1 Drug Delivery Systems for Intraoral Application

Intraoral drug delivery systems (Figure 5.1) are intended for the movement of drug through the oral mucosa. These systems generally fall into one of four broad categories: mucoadhesive buccal patches and tablets, quick disintegrating solid dosage forms, solid intraoral delivery systems and aerosol intraoral drug delivery systems.

5.1.2 Quick Dissolving Novel Pharmaceutical Films/Wafer Dosage Form

Quick dissolving pharmaceutical oral films/wafers are an attractive route of administration because they dissolve or deaggregate spontaneously in the oral cavity, resulting in a solution or suspension without water. Effectively it is a solid-dosage form providing the convenience of a liquid-dosage form. The perception of using thin films

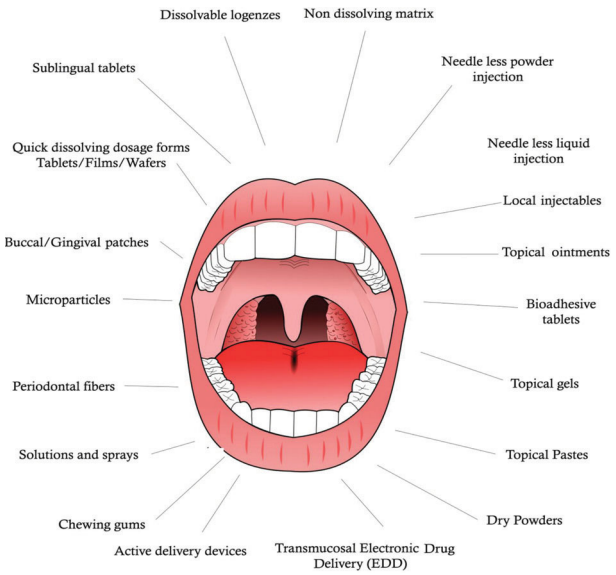


Figure 5.1 Types of intraoral dosage forms [4].

or strips for delivery of drugs into the mouth is not novel; reports exist of delivery of lidocaine, a local anesthetic, for dental applications from polymer films [5]. These films generally dissolve rapidly (within seconds) to release the active agents, but can be tailored to release the drug more slowly as well, depending upon their thickness and selection of the polymer matrix. A film or strip can be defined as a dosage form employing a water-dissolving polymer (like a hydrocolloid, which may be a bioadhesive polymer), allowing the dosage form to quickly hydrate, adhere, and dissolve when placed on the tongue or in the oral cavity (i.e., buccal, palatal, gingival, lingual, or sublingual, etc.) to offer rapid local or systemic drug delivery. Drug release, depending on the rate of dissolution of the films, may be either quick (within seconds) or slower (within minutes). These films are monolithic matrices and release the active ingredients multidirectionally when placed in the oral cavity [4]. A majority of the drugs prescribed to patients are conventional tablets and capsules, and less attention has been paid to patients experiencing difficulty in swallowing (dysphagia) [6]. It has been reported that 35 percent of the general population, 30 to 40 percent of elderly nursing home patients, and 25 to 50 percent of patients hospitalized for acute neuromuscular disorders and head injuries have dysphagia. The main causes of dysphagia include esophageal disorders such as achalasia, gastroesophageal reflux disease (GERD), cardiovascular conditions such as aneurysms, autoimmune diseases such as Sjogren's syndrome and acquired immune deficiency syndrome (AIDS), thyroid surgery, radiation therapy to head and neck or oral cavity, and other neurological diseases such as cerebral palsy [7]. Oral wafers/oral disintegrating films (ODFs), postage stamp-sized strips of thin polymeric films, are relatively new dosage forms for this route of administration. They are intended to disintegrate or dissolve almost instantaneously when placed onto the buccal cavity [8]. The pharmaceutical wafers holds potential advantages like rapid disintegration, no swallowing or chewing, no coadministration of water, accurate dosing compared to liquid products, greater safety and efficacy, along with patient compliance. Wafers are intended to dissolve rapidly in the saliva without swallowing the whole dosage form, making them the right choice for pediatric and geriatric patients, bedridden patients or patients suffering from dysphagia, Parkinson's disease, mucositis or vomiting [9].

The first type of non-medicated oral quick dissolving film that came out on the consumer market was Listerine® PocketPaks™ breath freshener in October 2001, a product of Pfizer's Warner-Lambert Consumer Healthcare Division [10]. With this launch, a number of other innovative thin-film products have also found their way into the consumer market for breath freshening like FreshBurst™, Myntz™ Instastripz, Gel-A-Mint Sugar Free™, MagikStrips™, Altoids™ Strips and many more.

Prestige Brands International introduced the first oral thin-film product to incorporate a drug, Chloraseptic® Relief Strips™, in the United States in September 2003, for relief of sore throat [11]. Zengen, Inc. was behind the development of this new delivery technology of a medicated oral strip structured as a proprietary bilayer system. The system contains 3 mg of benzocaine and 3 mg of menthol (oral anesthetics and analgesics) and is made available in tamper-evident blister packs. Chloraseptic Relief Strips include the following inactive ingredients: acesulfame potassium, hydrocolloids (carboxymethylcellulose and modified pectin), flavoring agent (cherry flavor), plasticizer (glycerin), sweetener (sucralose), coloring agent (Red 40), lecithin, magnesium silicate, and water.

A number of companies have been involved as innovators in the development and manufacturing of patented thin-film strip technologies including LTS Lohmann Therapie-Systeme AG and Lavipharm Laboratories, which developed the Quick-Dis™ and Slow-Dis™ technologies [12].

5.1.3 Buccoadhesive Wafer Dosage Form Advantages over Conventional Oral Dosage Forms

The specific advantages that the buccoadhesive wafer dosage form has over conventional oral dosage forms are:

- A fast wetting phenomenon, which helps them to adhere to the mucosa and/or dissolve rapidly, avoiding easy spitting out.
- The fear of choking or inhalation makes patient incompliance to orally disintegrating tablets. ODF/wafer technology completely eliminates these problems [8].
- As they are thin and can be taken without water, wafers are ideal for travelers or patients who do not have continuous access to water.
- Lyophilization is a common process for manufacturing orally disintegrating tablets. However, the manufacturing of ODFs stands on the technology for producing transdermal patches, which is less expensive than lyophilization [8].
- Convenient accurate dosing is achieved in the case of ODFs, which makes them superior to liquid formulations such as drops or syrups [9].
- As drug release from wafers into the oral cavity requires a few seconds, a rapid onset of action could be achieved.
- As the drug is absorbed through the oral mucosa, first-pass metabolism for some drugs is avoided, improving their bioavailability. Buccal absorption may be particularly beneficial, for example, for patients suffering from migraines, runny noses and allergic rhinitis.
- There are no known adverse physiological effects of ODFs, and the oromucosa is less vulnerable to damage or irritation than the other mucosa.

5.2 Preparation Methods of Novel Quick Dissolving Films

Generally, novel quick dissolving films are made by some of the established technologies like coating, solvent casting and hot-melt extrusion process. At first a wide web is produced and afterwards it will be cut into the desired size needed by the dosage form.

5.2.1 Hot-Melt Extrusion Process

Hot-melt extrusion has been approved as a solvent-free manufacturing process for ODFs. In this process, the API and other ingredients are mixed in a dry state, which is then subjected to a heating process and extruded out in a molten state. The blend of API, polymers

and other excipient is heated and pressed through a slot nozzle to a web. The web is then cooled down and cut to film size [13]. Recently, another method was reported in the literature which used hot-melt extrusion and spherical dies. The thin web is prepared by using a cooled roll. In this process, solvents are completely eliminated [2]. The strips, formed by this, are further cooled and cut to the desired size. The thermolabile APIs may degrade at the high temperature used in this process. Hence, the literature reveals that the solvent casting method is the method of choice for manufacturing of quick dissolving films.

5.2.2 Solvent Casting Method

Typically this technique includes the preparation of the base material that involves the blending of film-forming excipients and therefore the API mixed along in a very appropriate solvent or solvent system. The choice of solvent basically depends on the API to be incorporated into the film/strip. The physicochemical properties of the API like heat sensitivity, shear sensitivity, the polymorphic mode of the API utilized, compatibility of the API with solvent and different strip excipients are to be critically studied. The several components during this are liquid rheology, desired mass to be forged and content uniformity. Solvents used for the preparation of solution or suspension ought to ideally be elite ones from the ICH 3 solvent list [2].

The heating method could also be enclosed at this stage for the entire dissolution of materials. At this time, the necessary point to be considered is the air bubbles which will have become entrapped throughout the solution preparation. Entrapped air might tend to provide uneven strips. A deaeration step is imperative to induce a strip with uniform thickness. Vacuum motor-assisted machines will be utilized to get rid of the entrapped air. Several corporations adopt bubble-free mixing treatment having the appropriate kind of specialized stirring systems. Another necessary side is the entrapped moisture within the solution. This could attribute changes within the mechanical properties of the strips like enduringness, flexibility, folding endurance, modulas of elasticity, elongation, etc. Therefore, care ought to be exercised by using appropriate moisture controls within the production space. The solution is subjected to continuous intermixture method so as to retain the viscousness and concentration. Once this solution is ready, the film casting is performed whereby a strip of desired thickness is cast onto a moving inert substrate. Appropriate rollers are utilized for guiding the solution onto the substrate. The clearance or tolerance between the roller, and therefore the substrate, determines the specified thickness of the strip. The strip is then subjected to the drying method to get rid of the solvent. In R&D for small-scale production, film strips are sometimes cast using applicators. Adjustable film applicators enable the user to modulate the film thickness.

5.3 Polymers and Blends for Utilization in Different Quick Dissolving Films

The polymers and blends listed in the literature for utilization in quick dissolving films are presented in Table 5.1.

Table 5.1 Different polymeric platforms of drugs along with their method of preparation and characterization.

Polymeric Platform	Drug	Method of Preparation	Characterization	Reference
Maltodextrin (Glucidex®IT12, MDX)	Piroxicam	Casting method	1,2,3,6,7,8	[14]
Kollidon®VA 64 or Soluplus® with hydroxypropyl cellulose (HPC)	chlorpheniramine and indomethacin	hot-melt extrude	1,5,7, 8,11,12,13	[15]
HPMC and pullulan	Granisetron Hydrochloride	Solvent casting	1,2	[16]
Hydroxypropyl methylcellulose (HPMC) (Pharmacoat 606) with crospovidone (Kollidon CL –M)	rasagiline mesylate	Solvent casting with inkjet printing	10,13,14	[17]
Sodium carboxy methyl cellulose with Hydroxyl propylmethyl cellulose K15	Atenolol	Solvent casting	2,6,	[18]
Hydroxypropyl methylcellulose with microcrystalline cellulose and low substituted hydroxypropyl cellulose	Herpetrione in SDS and PVP K-30 to form nanosuspension	Solvent casting	1,13,14	[19]
Microcrystalline cellulose, Hydroxypropylmethyl cellulose, Polysorbate 80 and low-substituted Hydroxypropyl cellulose	dexamethasone	Coating	Pharmacokinetic study	[20]
Sodium CMC and Hydroxypropyl cellulose	loratadine	Solvent casting	1,7,8,9,13	[21]
Sodium alginate with Hydroxypropyl cellulose	loratadine	Solvent casting	1,7,8,9,13	[22]

1. Film thickness, 2. Film flexibility, 3.Tensile properties, 4. Tear resistance, 5. Young's modulus, 6. Stickiness determination, 7. ATR-FTIR spectroscopy, 8. Differential scanning calorimetry (DSC), 9. Disintegration test, 10. Contact angle measurement, 11. TGA, 12. Water content determination, 13. X-ray diffraction, 14. SEM.

5.4 Polymers in Novel Quick Dissolving Films

5.4.1 Hydroxypropyl Cellulose (Cellulose, 2-hydroxypropyl ether)

Hydroxypropyl celllulose is also known as cellulose, hydroxypropyl ether; E463; hydroxypropyl cellulosum; hyprolose; Klucel; Nisso HPC; and oxypropylated cellulose.

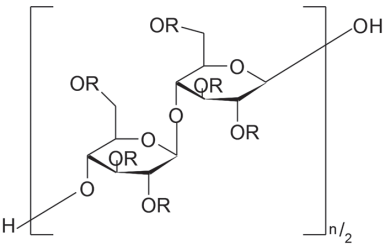
Hydroxypropyl cellulose (Scheme 5.1) is a stable material which is white to slightly yellow colored, odorless and a tasteless powder. It is commercially available in different grades having various solution viscosities. Depending upon the polymer grade, the viscosity of solutions ranges from 75 mPa s – 6500 mPa s. Its molecular weight has a range of 50,000–1,250,000. In regards to the melting point, the material softens at 130°C and chars at 260–275°C. A characteristic of Hydroxypropyl cellulose is that of absorbing moisture from the atmosphere. Quantity of water absorbed largely depends upon the initial moisture content, temperature and relative humidity of the surrounding air. Typical equilibrium moisture content values at 258°C are 4% w/w and 12% w/w at 50% and 84% relative humidity respectively.

5.4.1.1 Method of Preparation

Reacting a purified form of cellulose with sodium hydroxide produces a swollen alkali cellulose that is chemically more reactive than untreated cellulose. At elevated temperature and pressure, the alkali cellulose is then reacted with propylene oxide. Substitution of the propylene oxide can be done on the cellulose through an ether linkage at the three reactive hydroxyls present on each anhydroglucose monomer unit of the cellulose chain. The process of etherification takes place in such a way that hydroxypropyl substituent groups contain almost entirely secondary hydroxyls. Secondary hydroxyl present in the side chain is available for further reaction with the propylene oxide, and 'chaining-out' may take place. Results include the formation of side chains containing more than 1 mole of combined propylene oxide.

5.4.1.2 Pharmaceutical Application

Hydroxypropyl cellulose acts as a tablet binder in the range of 2–8% of tablet weight. Its most suitable applicability is for water-soluble drugs. This polymer is used for



R=H or $[CH_3CH(CH_3)O]m$
m= Common Integral number of Cellulose derivative
Hydroxypropyl cellulose

Scheme 5.1 Chemical structure of Hydroxypropyl cellulose.

preparation of modified release dosage form, for the preparation of microcapsules and as a thickening agent in oral and topical formulations. Due to its non-ionic nature, it is used as an emulsifier in cosmetic formulations. It can be used for the preparation of flexible films alone or in combination with Hypromellose, as it imparts low surface and interfacial tension to its solution.

5.4.1.3 Safety and Regularity Status

This polymer is generally considered as a nontoxic and non-irritant material. It is GRAS listed and included in the FDA Inactive Ingredient Guide.

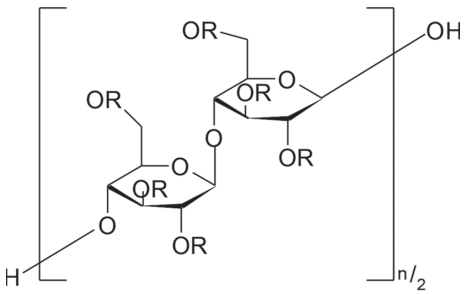
5.4.2 Hydroxypropyl Methyl Cellulose (Cellulose Hydroxypropyl Methyl Ether)

Hydroxypropyl methyl cellulose (Scheme 5.2) is also known as Benecel MHPC; E464; hydroxypropyl methylcellulose; HPMC; hypromellosum; Methocel; methylcellulose propylene glycol ether; methyl hydroxypropylcellulose; Metolose; MHPC; Pharmacoat; Tylopur; and Tylose MO.

Hypromellose is an odorless, tasteless and white or creamy white fibrous or granular powder. The molecular weight varies between 10000–1500000. It absorbs moisture from the atmosphere; the amount of water absorbed depends upon the initial moisture content, temperature and relative humidity of the surrounding air. It browns at 190–200°C; chars at 225–230°C. Glass transition temperature is 170–180°C.

5.4.2.1 Method of Manufacturing

The purified form of cellulose, obtained from cotton linters or wood pulp, is treated with sodium hydroxide solution to produce swollen alkali cellulose that is chemically more reactive than untreated cellulose. Reaction of the alkali cellulose with chloromethane and propylene oxide produces methyl hydroxypropyl ethers of cellulose. Further purification of the fibrous reaction product is done and is grounded to fine, uniform powder or granules.



R= H or CH_3 or $CH_3CH(OH)CH_2$

Hydroxypropyl methyl cellulose

Scheme 5.2 Chemical structure of Hydroxypropyl methyl cellulose.

Production of low viscosity grades is done by exposing Hypromellose to anhydrous hydrogen chloride to induce depolymerization.

5.4.2.2 Pharmaceutical Application

Hypromellose is widely used in oral, ophthalmic and topical formulations; used primarily as a tablet binder, film coating agent, film-forming agent and as a matrix for use in extended release formulations. It is also used as a suspending and thickening agent. In gels and ointments, it is also used as an emulsifier, suspending agent and stabilizing agent.

Hypromellose is used to manufacture capsules, as an adhesive in plastic bandages and as a wetting agent in contact lenses.

5.4.2.3 Safety and Regulatory Status

Hypromellose is listed in GRAS and included in FDA Inactive Ingredient Guide.

5.4.3 Pullulan

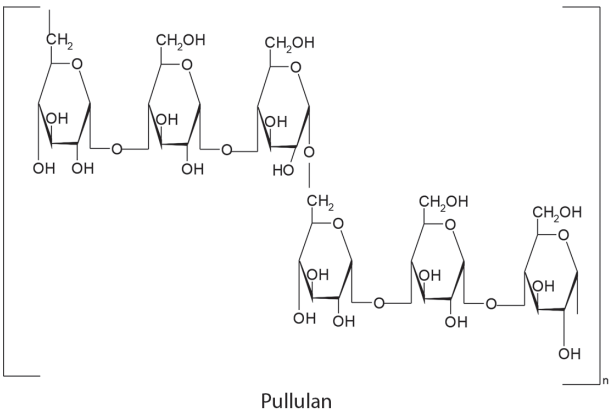
Chemically, Pullulan is 1, 6α-linked maltotriose (Scheme 5.3). It is available as white, odorless, tasteless, stable powder. The molecular weight ranges from 8000–2,000,000. It is soluble in hot and cold water. The viscosity (10%w/w, 30°C) of Pullulan is found to be 100–180 mm^2/s and melting point is 107°C. Moisture content is less than 6 %w/w.

5.4.3.1 Method of Manufacturing

Production of Pullulan occurs from starch by the fungus *Aureobasidium pullulans.*

5.4.3.2 Pharmaceutical Application

Pullulan is used extensively in the food industry to provide bulk and texture. Hydrophobic grades of Pullulan are used for preparation of nanoparticles for targeted delivery. Pullulan can also be used as a replacement for dextran as a plasma expander.



Pullulan

Scheme 5.3 Chemical structure of Pullulan.

Pullulan films are strong and are therefore used for decoration of food products, in confectionaries. Pullulan acts as an ideal carrier system for flavors, colors and drugs.

Pullulan is used in coating for immediate release tablets and it is also used for preparation of capsule shells. An edible, mostly tasteless polymer, its chief commercial use is in the manufacture of edible films that are used in various breath freshener or oral hygiene products such as Listerine Cool Mint by Johnson & Johnson (USA).

5.4.3.3 Safety and Regulatory Status

Pullulan is listed in GRAS.

5.4.4 Carboxymethyl Cellulose

Carboxymethyl cellulose is commonly known as Akulell, Blanose, Aquasorb, and CMC sodium (Scheme 5.4).

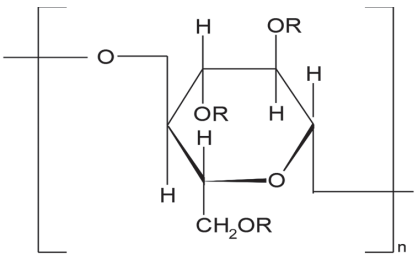
It is a white, odorless powder, having a molecular weight of 90,000–700,000, and is easily dispersed in water to form a clear or colloidal solution.

5.4.4.1 Pharmaceutical Application

Carboxymethyl cellulose is used widely in oral and topical formulations. It is used mainly as a consistency increasing agent. It is used as a stabilizer for preparation of suspensions and emulsions. Carboxymethyl cellulose can be used as a binder or disintegrant depending on the grade and concentration used in the formulation. It is also reported as a cryoprotective agent. It has a mucoadhesive property which is used in a number of topical and also oral preparations. It is reported for use together with other film-forming polymers for preparation of oral films or for coating tablets. It can be used for preparation of microparticles because it forms complex coacervates with gelatin and pectin.

5.4.4.2 Safety and Regulatory Status

Carboxymethyl cellulose is GRAS listed.



R=H or CH_2COONa

Sodium carboxy methyl cellulose

Scheme 5.4 Chemical structure of Sodium carboxymethyl cellulose.

5.4.5 Polyvinyl Pyrollidone

Polyvinyl pyrollidone is also known as E1201; Kollidon (Scheme 5.5); Plasdone; poly[1-(2-oxo-1-pyrrolidinyl)ethylene]; polyvidone polyvinylpyrrolidone; povidonum; Povipharm; PVP; and 1-vinyl-2-pyrrolidinone polymer.

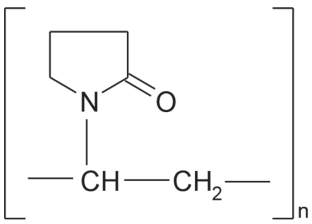
Chemically it is a 1-Ethenyl-2-pyrrolidinone homopolymer. It is categorized as non-ionic polymer.

The USP 32 describes povidone as a synthetic polymer consisting essentially of linear 1-vinyl-2-pyrrolidinone groups, the differing degree of polymerization of which results in polymers of various molecular weights. It is characterized by its viscosity in aqueous solution, relative to that of water, expressed as a K-value, in the range of 10–120. Approximate molecular weights for different grades of povidone are presented in Table 5.2.

Povidone occurs as a fine, white to creamy-white colored, odorless or almost odorless, hygroscopic powder. Povidones with K-values equal to or lower than 30 are manufactured by spray-drying and occur as spheres. Povidone K-90 and higher K-value povidones are manufactured by drum-drying and occur as plates. It softens at 150°C.

5.4.5.1 Pharmaceutical Applications

Though povidone is used in a variety of pharmaceutical formulations, it is primarily used in solid-dosage forms. In tableting, povidone solutions are used as binders in wet-granulation processes. Povidone is also added to powder blends in the dry form and granulated *in situ* by the addition of water, alcohol, or hydroalcoholic solutions. Povidone is used as a solubilizer in oral and parenteral formulations, and has been shown to enhance dissolution of poorly soluble drugs from solid-dosage forms.



Kollidon

Scheme 5.5 Chemical structure of Kollidon.

Table 5.2 Approximate molecular weights for different grades of povidone.

K-Value	Approximate Molecular Weight	K-Value	Approximate Molecular Weight
12	2500	30	50000
15	8000	60	400000
17	10000	90	1 000 000
25	30000	120	3 000 000

Povidone solutions may also be used as coating agents or as binders when coating active pharmaceutical ingredients on a support such as sugar beads. Additionally, povidone is used as a suspending, stabilizing, or viscosity increasing agent in a number of topical and oral suspensions and solutions.

5.4.5.2 Method of Preparation

Povidone is manufactured by the Reppe process. Acetylene and formaldehyde are reacted in the presence of a highly active copper acetylide catalyst to form butynediol, which is hydrogenated to butanediol and then cyclodehydrogenated to form butyrolactone. Pyrrolidone is produced by reacting butyrolactone with ammonia. This is followed by a vinylation reaction in which pyrrolidone and acetylene are reacted under pressure. The monomer, vinylpyrrolidone, is then polymerized in the presence of a combination of catalysts to produce povidone.

5.4.5.3 Safety and Regulatory Status

Polyvinyl pyrollidone has been accepted for use in Europe as a food additive. It is also included in the FDA Inactive Ingredients Database; in non-parenteral medicines licensed in the UK and in the Canadian List of Acceptable Non-medicinal Ingredients.

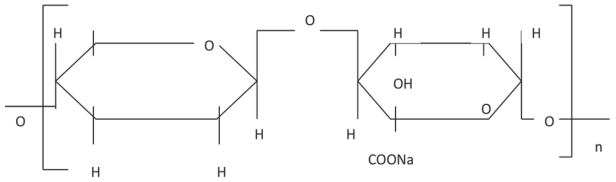
5.4.6 Sodium Alginate

Sodium alginate (Scheme 5.6) is also known as Alginatosodico; algin; alginic acid, sodium salt; E401; Kelcosol; Keltone; natriialginas; Protanal; and sodium polymannuronate.

It consists chiefly of the sodium salt of alginic acid, which is a mixture of polyuronic acids composed of residues of D-mannuronic acid and L-guluronic acid. It occurs as an odorless and tasteless, white to pale yellowish-brown colored powder.

5.4.6.1 Pharmaceutical Applications

Sodium alginate is used in a variety of oral and topical pharmaceutical formulations. In tablet formulations, it may be used as both a binder and disintegrant; it has been used as a diluent in capsule formulations. Since it can delay the dissolution of a drug from tablets, capsules, and aqueous suspensions, it has been used in the preparation of sustained release oral formulations. In topical formulations, sodium alginate is widely used as a thickening and suspending agent in a variety of pastes, creams, and gels, and



Scheme 5.6 Chemical structure of Sodium alginate.

as a stabilizing agent for oil-in-water emulsions. The adhesiveness of hydrogels prepared from sodium alginate has been investigated, and drug release from oral mucosal adhesive tablets, buccal gels, and vaginal tablets based on sodium alginate have been reported. The esophageal bioadhesion of sodium alginate suspensions may provide a barrier against gastric reflux or site-specific delivery of therapeutic agents. Other novel delivery systems containing sodium alginate include ophthalmic solutions that form a gel *in situ* when administered to the eye; and *in-situ* forming gel containing paracetamol for oral administration; nasal delivery systems based on mucoadhesive microspheres; and a freeze-dried device intended for the delivery of bone-growth factors.

5.4.6.2 Method of Preparation

Sodium alginate is formed when Alginic acid is extracted from brown seaweed and is neutralized with sodium bicarbonate.

5.4.6.3 Safety and Regulatory Status

Sodium alginate is GRAS listed and is accepted in Europe for use as a food additive. It is also included in the FDA Inactive Ingredients Database.

5.4.7 Polymethacrylates

Polymethacrylates are also known as Acryl-EZE; acidi methacrylici et ethylis acrylatis polymerisatum; acidi methacrylici et methylis methacrylatis polymerisatum; ammonio methacrylatis copolymerum; copolymerum methacrylatis butylati basicum; Eastacryl; Eudragit; Kollicoat MAE; polyacrylatis dispersio 30 per centum; and polymeric methacrylates. The chemical and trade names of polymethacrylates are listed in Table 5.3, along with the names of the companies that produce them.

PhEur 6.2 describes methacrylic acid-ethyl acrylate copolymer (1:1) as a copolymer of methacrylic acid and ethyl acrylate having a mean relative molecular mass of about 2, 50,000. The ratio of carboxylic groups to ester groups is about 1:1. It may contain suitable surfactants such as sodium dodecyl sulfate or polysorbate 80. An aqueous 30% w/v dispersion of this material is also defined in a separate monograph. As described in PhEur 6.0, Methacrylic acid-methyl methacrylate copolymer (1:1) is a copolymer of methacrylic acid and methyl methacrylate having a mean relative molecular mass of about 1, 35,000. The ratio of carboxylic acid to ester groups is about 1:1. A further monograph in PhEur 6.0 describes methacrylic acid-methyl methacrylate copolymer (1: 2), where the ratio of carboxylic acid to ester groups is about 1:2. PhEur 6.0 describes basic butylatedmethyacrylate copolymer as a copolymer of (2-dimethylaminoethyl) methacrylate, butyl methyacrylate, and methyl methacrylate having a mean relative molecular mass of about 1, 50,000. The ratio of (2-dimethylaminoethyl)methacrylate groups to butyl methyacrylate and methyl methacrylate groups is about 2:1:1. The Ph Eur 6.0 describes ammoniomethyacrylate copolymer as a poly(ethyl propenoate-co-methyl 2-methylpropenoate-co-2-(trimethylammonio) ethyl 2-methylpropenoate) chloride having a mean relative molecular mass of about 1, 50,000. The ratio of ethyl propenoate to methyl 2-methylpropenoate to 2-(trimethylammonio) ethyl 2-methylpropenoate is

Table 5.3 Chemical and trade names of Polymethacrylates produced by various companies.

Chemical Name	Trade Name	Company Name
Poly(butyl methacrylate, (2-dimethylaminoethyl) methacrylate, methyl methacrylate) 1 : 2 : 1	Eudragit E 100 Eudragit E 12.5 Eudragit E PO	Evonik Industries
Poly(ethyl acrylate, methyl methacrylate) 2 : 1	Eudragit NE 30 D Eudragit NE 40 D Eudragit NM 30 D	Evonik Industries
Poly(methacrylic acid, methyl methacrylate) 1 : 1	Eudragit L 12.5 Eudragit L 12.5 P	Evonik Industries
Poly(methacrylic acid, ethyl acrylate) 1 : 1	Acryl-EZE Acryl-EZE 93A Acryl-EZE MP Eudragit L 30 D-55 Eudragit L 100-55 Eastacryl 30D Kollicoat MAE 30 DP Kollicoat MAE 100 P	Colorcon Colorcon Colorcon Evonik Industries Evonik Industries Eastman Chemical BASF Fine Chemicals BASF Fine Chemicals
Poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) 1 : 2 : 0.2	Eudragit RL 100 Eudragit RL PO Eudragit RL 30 D Eudragit RL 12.5	Evonik Industries
Poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) 1 : 2 : 0.1	Eudragit RS 100 Eudragit RS PO Eudragit RS 30 D Eudragit RS 12.5	Evonik Industries

about 1:2: 0.2 for Type A and 1:2: 0.1 for Type B. Polyacrylate dispersion (30 percent) is described in PhEur 6.3 as a dispersion in water of a copolymer of ethyl acrylate and methyl methacrylate having a mean relative molecular mass of about 8, 00,000. It may contain a suitable emulsifier. The USP32–NF27 describes methacrylic acid copolymer as a fully polymerized copolymer of methacrylic acid and an acrylic or methacrylic ester. The monograph defines three types of copolymers, namely, Type A, Type B, and Type C. They vary in their methacrylic acid content and solution viscosity. Type C may contain suitable surface-active agents. Ammonio methacrylate copolymers Type

A and Type B, consisting of fully polymerized copolymers of acrylic and methacrylic acid esters with a low content of quaternary ammonium groups, are also described in USP32–NF27. Variation occurs in their ammonio methacrylate units. USP32–NF27 also describes amino methacrylate copolymer as a fully polymerized copolymer of 2-dimethylaminoethyl methacrylate, butyl methacrylate and methyl methacrylate. Typically, the molecular weight of the polymer is >100 000.

5.4.7.1 Method of Manufacture

Polymerization of acrylic and methacrylic acids or their esters leads to its preparation, e.g., butyl ester or dimethylaminoethyl ester.

5.4.7.2 Safety and Regulatory Status

Polymethacrylates are included in the FDA Inactive Ingredients Database.

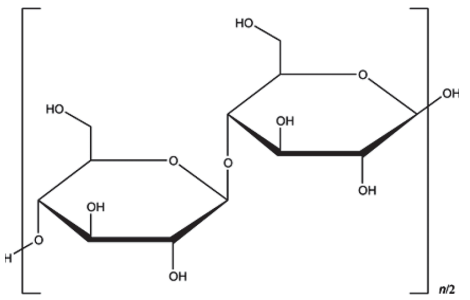
5.4.8 Microcrystalline Cellulose

Microcrystalline cellulose (Scheme 5.7) is also known as Avicel PH; Cellets; Celex; cellulose gel; hellulosum microcristallinum; Celphere; Ceolus KG; crystalline cellulose; E460; Emcocel; Ethispheres; Fibrocel; MCC Sanaq; Pharmacel; Tabulose; and Vivapur.

It is a purified, partially depolymerized cellulose, that occurs as a white, odorless, tasteless, crystalline powder and is composed of porous particles having an empirical formula $(C_6H_{10}O_5)_n$ and a molecular weight »36000. It is commercially available in different particle sizes and moisture grades that have different properties and applications. It chars at 260–270°C.

5.4.8.1 Pharmaceutical Applications

Microcrystalline cellulose is widely used in pharmaceuticals, primarily as a binder/diluent in oral tablet and capsule formulations where it is used in both wet-granulation and direct-compression processes. In addition to its use as a binder/diluent, microcrystalline cellulose also has some lubricant and disintegrant properties that make it useful in tableting. It acts as a disintegrant at 5–15% concentration.



Scheme 5.7 Chemical structure of Microcrystalline cellulose.

5.4.8.2 Method of Manufacturing

Controlled hydrolysis with dilute mineral acid solutions of α-cellulose, which is obtained as a pulp from fibrous plant materials, is used in the manufacturing of microcrystalline cellulose. Following hydrolysis, the hydrocellulose is purified by filtration and the aqueous slurry is spray-dried to form dry, porous particles of a broad size distribution.

5.4.8.3 Safety and Regulatory Status

Microcrystalline cellulose is listed in GRAS and is accepted for use as a food additive in Europe. It is also included in the FDA Inactive Ingredients Database.

5.5 Role of Plasticizers in Novel Quick Dissolving Film

Plasticizer is an important ingredient of the OS formulation. It helps to enhance the pliability of the strip and reduces the crispness of the strip. Plasticizer considerably improves the strip properties by reducing the glass transition temperature of the polymer. The choice of plasticizer depends upon its compatibility with the compound and conjointly the kind of solvent utilized within the casting of the strip.

Use of plasticizer can increase the flow of polymer and enhance the strength of the polymer [31,32]. As wafers still have a comparatively high water content once dried, water itself acts as plasticizer. Glycerol, propylene glycol, low molecular weight polythene glycols, phthalate derivatives like dimethyl, diethyl and dibutyl phthalate, citrate derivatives like tributyl, triethyl, ethanoyl group, triacetin and castor oil are some of the normally used plasticizer excipients. Generally the plasticizers are employed in a concentration of 0–20%w/w of dry polymer weight. But inappropriate use of plasticizer could result in film cracking, ripping and peeling of the strip [2]. It has been conjointly reported that the utilization of certain plasticizers may additionally have an effect on the absorption rate of the drug [33]. The plasticizer utilized ought to impart permanent flexibility to the strip and it depends on the volatile nature and also the form of interaction with the polymer. It ought to be noted that the properties of plasticizer are vital to decrease the glass transition temperature of polymer within the range of 40–60°C for non-aqueous solvent system and below 75°C for aqueous systems [34]. Plasticizer should be compatible with the drug in addition to alternative excipients used for preparation of the strip.

Among the various grades of polyethylene glycol (PEG); PEG 300 was found to be higher plasticizer for gelatin as compared to higher relative molecular mass PEG. This is often because lower molecular weight PEG formed visually superior films and had low water vapor permeation rate. Once sugars like mannitol and sorbitol were tested as plasticizers for gelatin strips, sorbitol was found to be higher as compared to mannitol since mannitol crystallizes out from the gelatin strip [35]. Maltodextrin can even be plasticized and regenerated into OS with incorporation of glycerol and propylene glycol as plasticizer within the concentration range of 16–20% w/w. In this case, glycerol was found to be higher than propylene glycol once the strips were manufactured by solvent casting as well as hot-melt extrusion strategies. However, PEG has miscibility issues with maltodextrins and does not act very well as good plasticizers

[14]. Certain drug molecules themselves will act as plasticizer. For instance, ibuprofen interacts with Eudragit RS 30 D in the role of a plasticizer. In this case, the glass transition temperature of Eudragit RS 30 D decreased and smooth film formation was ascertained as a result of the hydrogen bonding between the drug and the polymer. Also, the dissolution rate of ibuprofen decreased once its concentration within the formulation was increased [36].

There are two mechanisms propagated on how the plasticization takes place, specifically internal plasticization (involving chemical interaction) and external plasticizing impact. Formulators prefer to adopt the latter mechanism because it does not involve chemical interactive alterations within the product. An example of internal plasticization is wherever PEG 4000 was used as plasticizer for phenobarbital where the drug release was reduced to a significant extent [37]. The chemical structure and concentration of plasticizers play a crucial role in alleviating the glass transition temperature of the polymers. Cellulosic hydrophilic polymers were simply plasticized with hydroxyl group containing plasticizers like PEG, propylene glycol, glycerine and polyols. In contrast, less hydrophilic cellulosic polymers were plasticized with esters of citric acid and phthalic acid [8]. Glycerine acts as a better plasticizer for polyvinyl alcohol, whereas diethylene glycol can be used for both Hypromellose as well as polyvinyl alcohol films [37]. The literature conjointly witnesses that the taste of glycerine plasticized ODFs was preferred over the taste of propylene glycol plasticized films [14].

5.6 Characterization Procedure Listed in the Literature for Fast Dissolving Films

Different pharmacopoeias reveal the monographs of common dosage forms (e.g., Ph.Eur, USP). Though the dosage forms for application in oral cavity, i.e., medicated chewing gums, oromucosal preparations, orodispersible tablets or oral lyophillisates are enclosed, monographs and specifications for oral films or wafers have nevertheless been established. To decide the potency and dependableness of the pharmaceutically developed wafers, they are evaluated for their physicochemical parameters, which are of overriding importance. The characterizations of the various properties of fast dissolving films are categorized into mechanical, chemical and analytical properties.

5.6.1 Thickness and Weight Variation

The measurement of the thickness of the film is usually performed by using a well-calibrated electronic digital micrometer, screw gauge, vernier caliper or by SEM images. The essentiality of the measurement is to ascertain the uniformity of the film thickness, as it is directly related to the accuracy of dose in the film. Moreover, an optimum thickness is necessary to provide adequate bioadhesion [38]. The expansion of the thickness of the film has led to the criticalness of the choice of polymers (polyvinyl pyrrolidone, Eudragit, etc.) [14]. As a general criterion, an ideal buccal film should exhibit a thickness between 50 and 600 μm.

Weight variation technique includes weighing of the individual films followed by calculation of the average weights. Then the average weight of the films is subtracted from the individual weights. Large variation in weight depicts the inefficiency of the method employed and is likely to have non-uniform drug content.

5.6.2 Film Flexibility

The film flexibility is determined by adapting the ASTM bend mandrel test (D 4338-97), where a 2-3 cm sample is bended over an 8 mm mandrel and examined for cracks over the area of the bend in a strong light. If no cracks are visible at 5x magnification, the films are assumed to be flexible [14].

5.6.3 Tensile Strength

Mechanical properties play a crucial role in the physical integrity of the dosage form. The tensile strength measures the strength of the film as diametric tension or tearing force. Ongoing studies use this parameter to measure the mechanical strength of the films during formulation optimization. Stretching is done until the sample under test tears and the stress needed represents the tensile strength. It is calculated by dividing the force (N) at which the film breaks with the cross-sectional area (m^2) of the film.

The determination of the mechanical properties of a buccal film is usually based on the ASTM D882 method [39].

The literature reveals several methods for the measurement of tensile strength. Texture analyzer equipment, texture analyzer AG/MC1 (Acquati, I), is reported, which is equipped with a 5 N load to determine the tensile strength of the prepared film [14]. On the texture analyzer, each test strip is placed in the tensile grips longitudinally. Initial grip separation and crosshead speeds are 60 mm and 500 mm min^{-1} respectively. The conclusion of the test is drawn at the film break. In another method, Palem *et al.* [40] used a microprocessor-based advanced force gauze with a motorized test stand to assess the tensile strength of the buccal patches. It is calculated by the applied load at rupture divided by the cross-sectional area of the strip as given in the equation below [2]:

$$\text{Tensile strength} = [\text{Load at failure} \times 100]/ \text{Strip thickness Strip width} \quad (5.1)$$

5.6.4 Tear Resistance

Tear resistance of plastic film or sheeting is a complex function of its ultimate resistance to rupture. Basically, very low rate of loading—51 mm (2 in.)/min—is employed and is designed to measure the force to initiate tearing. The maximum stress or force (that is generally found near the onset of tearing) required to tear the specimen is recorded as the tear resistance value in newtons (or pounds-force) [41,42].

5.6.5 Young's Modulus

Measurement of stiffness of film represents Young's or elastic modules. The methods used for the measurement of tensile strength can also be utilized here. This measures resistance to deformation and can be observed by plotting the stress-strain curve wherein the slope measures the modulus. The higher the slope, the greater is the tensile modulus. However, a gentle slope measures a low tensile modulus and a case of deformation. Further, if the modulus keeps on changing with stress, the initial slope gives the modulus, and is commonly observed in the case of plastic materials. In general, films which are hard and brittle possess higher tensile strength and higher Young's modulus values [38].

5.6.6 Folding Endurance

The flexibility of buccal films is an important physical characteristic needed for easy application on the site of administration. This flexibility is measured quantitatively in terms of folding endurance and is determined by repeatedly folding the film at 180° angle of the plane at the same plane until it breaks or is folded to 300 times without breaking. The number of times the film is folded without breaking is computed as the folding endurance value.

5.6.7 ATR-FTIR Spectroscopy

An intact contact angle provides interaction between the drug and polymer in films. Hence the prepared films are generally assessed for this interaction. Analytical techniques such as FTIR can easily detect the chemicals and assess the compatibility of drug with the excipients by scanning the mixture. The FTIR spectrophotometer records the spectra of the samples using the KBr disc. The drug and excipients are pulverized, gently triturated with KBr powder in a specific weight ratio and compression is done using a hydrostatic press at a particular pressure. Then the disc is placed in the sample holder and scanned to obtain the spectra. In an advanced ATR-FTIR machine, a simple probe is used to record the spectra. The films are placed in contact with the probe and the machine thus can record the spectra.

5.6.8 Thermal Analysis and Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter records the thermograms of the samples, which provide insight into the state of the drug molecules inside the film. Any shift in the endothermic or exothermic peak or widening of peak area directly represents phase transition, recrystallization or molecular interaction of the drug molecule entrapped inside the film. Assessment of this is done by heating the sample in an aluminum pan at room temperature to elevated temperature (~500°C) at a specified heating rate (~10°C/min).

5.6.9 Disintegration Test

Deaggregation of solid dosage form (e.g., tablet or capsule) into its primary particles is termed as disintegration, and is thereby distinguished from drug dissolution. But in

case of oral quick dissolving film, disintegration and concurrent dissolution occurs in a small amount of saliva, making it difficult to mimic natural conditions and measure with an adequate method.

The disintegration time limit of 30 s or less for orally disintegrating tablets described in CDER guidance can be applied to fast dissolving oral strips [43]. The literature reveals different methods for determination of the disintegration process of quick dissolving films. Use of a pharmacopoeial disintegrating test apparatus is also recommended for such a study. A simple method as described by El-Setouhy [44] is by placing the film size required for dose delivery (2×2 cm) on a glass Petri dish containing 10 mL of distilled water. *In-vitro* disintegration time is recorded as the time required for the film to break. In another method, films are fixed in slide frames and placed upon a beaker. Placing a drop (200µl) of distilled water on the film and noting the time until the drop was able to wet through the film was recorded by Preis and coworkers [45].

Thermomechanical analysis for swelling is also studied to investigate the disintegration process. In the case of buccal wafers, the films swell and subsequently dissolve, so it is not a swelling process in the common sense. Thermomechanical analysis (TMA) is reported for buccal wafers using a Mettler TA 3000 apparatus (Mettler Toledo, Germany) with TC 10A processor and a TMA 40 load cell. Discs are film coated with either placebo or verum film solutions, each with the same width (900µm). A film-coated disc is placed into a crucible and annealed. The measuring sensor is placed onto the surface with a constant force of 0.02 N at a constant temperature of 37°C. Addition of 250 µL of purified water is done using a syringe [46]. Disintegration behavior is predicted from the developed setup. The swelling process is finished when the sensor dips onto the surface of the metallic disc.

Contact angle measurement is another type of process utilized for the study of disintegration time of quick dissolving films. Optical contact angle meter measures the time-dependent contact angles. Distilled water of volume 7.5 µL is dropped onto the film lying planar on the surface at room temperature. After 30 s the contact angle is determined by using the specialized software. Measurement of the contact angle can be done by different methods like the two tangential methods, a height–width ratio, the circle fitting and the sessile drop fitting [46].

5.6.10 X-ray Diffraction Study or Crystallinity Study of Films

An X-ray diffractometer can determine the physical form (crystalline or amorphous) of the drug molecule inside the film by X-ray crystallographic analyses. Placing the film in the sample holder and acquiring the XRD transmission diffractograms with a specific X-ray source over a start to end diffraction angle, scan range and scan speed can be obtained. The literature cites reports of identification of solubilization or conversion of crystalline drug to molecular dispersion within the films during the formulation stage by X-ray diffractograms, due to incorporation of plasticizers like propylene glycol [22].

5.6.11 Morphological Study

Surface properties, i.e., surface texture (smooth/rough), drug distribution (homogeneity, aggregated/scattered), thickness, etc., of prepared films can be evaluated

using SEM, electron microscopy and scanning tunneling microscopy. The literature reveals that most of the studies are reliant on SEM for assessing the surface morphology of the films. This is performed by mounting the films on stubs that are sputter coated with gold in an inert environment and photographed using SEM to obtain suitable magnified images. Identification of the shape, size and number of pores in the buccal films and the possible influence of plasticizer has been reported by the use of SEM. Certain studies have reported an assessment of the influence of chemical composition of the film on the surface morphology, crystallinity, etc., by utilization of SEM [38].

Analysis of the distribution of substances within the quick dissolving films or wafers is done by Near-infrared chemical imaging (NIR-CI). NIR-CI is a non-invasive approach where the polymers and the active ingredient are analyzed separately. Suitable vibrational bands for both substances are detected for selective imaging. During manufacturing of the quick dissolving films/wafers the recrystallization process takes place, and clarification of whether the polymer, the active ingredient or one of the excipients is recrystallized during this process is done by NIR-CI [46].

5.7 Conclusion and Future Perspectives

Many pharmaceutical companies are switching their product franchises from ODTs to quick dissolving films due to their advantages over ODTs with respect to their being a consumer-friendly alternative. Patent non-infringing product development is opened by utilization of these delivery systems. It also allows brand extension by the pharmaceutical companies for their existing products, and also helps to improve product lifecycle management by increasing the patent life of existing molecules or products. Due to ease of fabrication, bypassing complicated and expensive processes (like lyophilization) used to manufacture ODTs, these types of formulations reduce the overall cost of the therapy. This delivery platform shows promising business potential for the future in different areas of pharmaceuticals, cosmeceuticals and in nutraceuticals.

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6

Biomaterial Design for Human ESCs and iPSCs on Feeder-Free Culture toward Pharmaceutical Usage of Stem Cells

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Abstract
This chapter describes recent developments of natural and synthetic biomaterials to support the propagation of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), while maintaining pluripotency in feeder-free cultures. hPSCs are differentiated into any kind of tissue cells in our body, which will be used as pharmaceuticals as cell therapy. The development of methods for culturing these cells without using mouse embryonic fibroblasts (MEFs) as a feeder layer will enable more reproducible culture conditions and reduce the risk of xenogenic contaminants when used in pharmaceuticals. The combination of human ECM proteins or cell adhesion molecules and synthetic biomaterials with well-designed surfaces and/or structures (e.g., scaffolds, hydrogels, microcarriers, microcapsules, or microfibers) in the presence of a chemically defined medium containing recombinant growth factors would offer a xeno-free alternative to feeder cells for culturing hPSCs and maintaining their pluripotency.

Keywords: Embryonic stem cells, induced pluripotent stem cells, cell culture, biomaterial, pluripotency, differentiation, vitronectin, extracellular matrix

6.1 Introduction

Human embryonic stem cells (hESCs) are derived from the inner cell mass of 3- to 5-day-old blastocysts [1,3,4]. Recently, pluripotent stem cells that are similar to ESCs were derived from an adult somatic cell by the "forced" expression of certain pluripotency genes [5–8], such as Oct3/4, Sox2, c-Myc and klf-4, or their proteins

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[9,10] and microRNAs [11]. These cells are known as induced pluripotent stem cells (iPSCs). iPSCs are believed to be similar to ESCs in many respects. However, human iPSCs (hiPSCs) do not have the ethical concerns that hESCs have, which is due to the destruction of human embryo for the preparation of hESCs. Human pluripotent stem cells (hPSCs), such as hESCs and hiPSCs, open avenues for drug discovery and regenerative medicine. The development of a fully defined microenvironment for culturing hPSCs will have a tremendous impact on the use of hPSCs in cell therapy and tissue engineering [12–15]. The current gold standard for maintaining hPSCs in an undifferentiated state commonly uses the culturing method on feeder cells (e.g., mouse embryonic fibroblasts [MEFs] or human feeder cells, such as human foreskin fibroblasts [16,17] and human adipose-derived cells [18]) or on Matrigel [19,20] or Geltrex [21] (Figure 6.1). The use of feeder cells to culture hPSCs is time consuming, varies depending on the specific lot of feeder cells, and is labor intensive. In contrast, Matrigel and Geltrex are composed of substrates isolated from the sarcomas of Engelbreth-Holm-Swarm mice, which include collagen IV, laminin, heparan sulfate proteoglycans, enactin, and growth factors (e.g., fibroblast growth factor [FGF], epidermal growth factor [EGF], and transforming growth factor-β [TGF-β]), and have been used to maintain the pluripotency of many hPSC lines [1]. These culture conditions are undefined, and their xenogenic components of these molecules obstruct the clinical application of hPSCs [22,23]. Furthermore, there are concerns regarding the transfer of xenogenic viruses, in addition to immunogenic epitopes, such as the non-human sialic acid *N*-glycolylneuraminic acid (Neu5Gc), to humans [22].

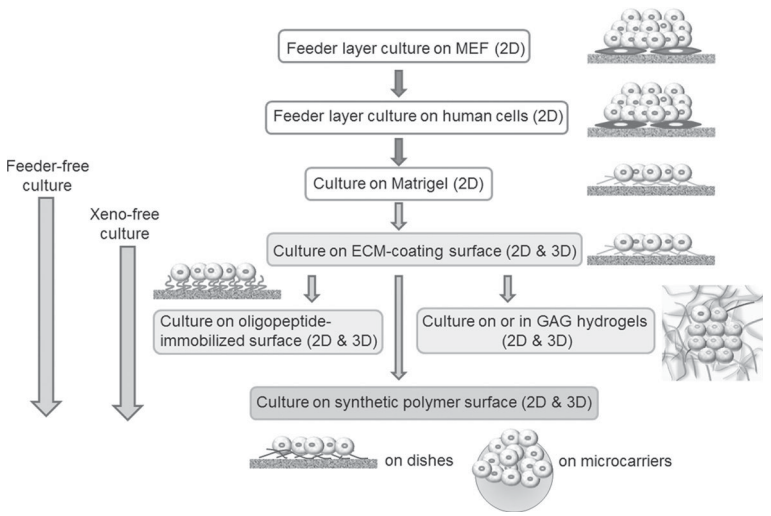


Figure 6.1 Culture methods for human pluripotent stem cells (hPSCs). hPSCs can be cultured on mouse embryonic fibroblasts (MEFs) or human feeder cells. Feeder-free culturing of hPSCs is possible on xeno-containing Matrigel. Several types of feeder-free and xeno-free cultures of hPSCs can be developed on extracellular matrix (ECM)-coated surfaces and oligopeptide-immobilized surfaces. hPSCs can also be cultured on polysaccharide hydrogels or on synthetic polymer surfaces by selecting a specific GAG or a specific polymer with optimal water content. Reproduced with permission from [2]; Copyright 2014 Elsevier Inc.

Table 6.1 Feeder-free culture of hPSCs on ECM-immobilized substrates in a defined medium.[a] Reproduced with permission from [2]. Copyright 2014 Elsevier Inc.

Cell Lines	Cell Culture Substrates (2D or 3D Culture)	Culture Medium	Longest Time in Culture	Ref (Year)
hESCs (H9, CA2)	Decellularized ECMs from human foreskin fibroblasts (2D)	HEScGRO with bFGF	20 passages	46 (2010)
hESCs (H1, H9)	Decellularized ECMs from human EB derived from hESCs (2D)	TeSR2	20 passages	43 (2011)
hESCs	Decellularized ECMs from human foreskin fibroblasts (2D)	TeSR2	not specified	44 (2012)
hiPSCs (UTA1)	FN-coating dishes (2D)	hESF9a	27 passages	61 (2010)
hESC (I6)	CELLstart-coating dishes (2D)	StemPro	28 passages	21 (2009)
hESCs (Shef3, Shef6)	CELLstart-coating dishes (2D)	StemPro	20 passages	51 (2011)
iPSC from ADSCs	CELLstart-coating dishes (2D)	NutriStem	Not specified	55 (2010)
hESCs (RC6, 9, 10, 13)	CELLstart-coating dishes (2D)	StemPro	not specified	56 (2012)
hESCs (H1, HSF1)	FN or LN-coating dishes (2D)	DMEM/F12 with small molecules & bFGF	25 passages	57 (2011)
hESCs (H1, H9, CA1) iPSC(4YA, 4YE, BJ-EOS, 4YF)	FN, VN, Col I , PDL, or CELLstart-coating dishes (2D)	HGM, XSR, or TeSR2 with and without Y27632	15–34 passages	31 (2012)
hESCs (H9, CHA6)	CELLstart or Vitronectin-coating dishes (2D)	mTeSR1 or StemPro	6 passages	58 (2010)

(*Continiued*)

Table 6.1 Cont.

Cell Lines	Cell Culture Substrates (2D or 3D Culture)	Culture Medium	Longest Time in Culture	Ref (Year)
hESCs (H9, CA1)	CELLstart, StemXVivo, BridgeECM, MEF-CMTX, or FN-coated dishes (2D)	mTeSR1	5-10 passages	53 (2011)
hESCs (HES2, HUES1)	VN, LN, FN, Col IV, or entactin-coating dishes (2D)	mTeSR1	8 passages	59 (2008)
hESCs (HS181, HS237, HS293, HS306)	Mixed ECMs (human Col IV, VN, FN, LN)-coating dishes (2D)	mTeSR1, X-Vivo 20	not specified	47 (2007)
hESC (BGO1V)	LN-coating dishes (2D)	DMEM/F12, Activin-A, bFGF, chimera protein	35 passages	70 (2010)
hESCs (HS207, HS420, HS401)	LN-511 coating dishes	O3 or H3 medium	20 passages	62 (2010)
hESCs (HES-3, H7)	LN or VN coating microcarriers (3D)	StemPro	20 passages	113 (2012)
hESCs (HES-3, H1)	VN-coating dishes	mTeSR1	20 passages	68 (2011)
hESCs (MEL1, MEL2, hES3)	Recombinant VN	StemPro	10 passages	69 (2010)

[a]Col, collagen; EB, embryonic body; FN, fibronectin; LN, laminin; VN, vitronectin; PDL, poly-D-lysine

There is a demand to develop hPSC lines suited to conditions that support large-scale cell manufacture under feeder-free and xeno-free conditions in facilities that are compliant with current good manufacturing practice (cGMP) [24–27]. The presence of feeder cells restricts the use of hESCs and hiPSCs. Extensive research has developed several alternative hPSC culture conditions that do not involve feeder cells. However, the key factors responsible for maintaining hPSC self-renewal capabilities are not still evident [19,28–30]. The basic FGF (bFGF), Wnt, and Activin/Nodal pathways are expected to be important for the maintenance of hPSC pluripotency. Furthermore, controlled propagation that maintains hPSC pluripotency is an important technology for creating the bankable populations of hPSCs required for future research geared toward regenerative medicine [28].

Table 6.2 Feeder-free culture of hPSCs on oligopeptide-immobilized, chimera protein-immobilized, and glycosaminoglycan-immobilized substrates in a defined medium.a Reproduced with permission from [2]. Copyright 2014 Elsevier Inc.

Cell Lines	Cell Culture Dubstrates (2D or 3D Culture)	Culture Medium	Longest Time in Culture	Ref (Year)
Synthetic oligopeptide- and polypeptide-immobilized surface				
hESCs (H1, H7, H9, H149), iPSCs (IMR90-1)	Oligopeptide (heparin-binding domain) immobilized dishes (2D)	mTeSR1 medium with ROCK inhibitor	17 passages	74 (2010)
hESCs (H1, H7)	Oligppeptide (BSP, VN)-immobilized dishes (2D)	XVIVO10 +GFs, mTeSR1	10 passages	71 (2010)
hESCs (H9, H14)	Cyclic RGD-immobilized dishes (2D)	mTeSR1	5 days	73 (2010)
hESCs (H9, BGN1)	PDL-immobilized dishes (2D)	mTeSR1 medium with ROCK inhibitor	2 passages	120 (2008)
hESCs (H1, H9)	PDL-coating pDTEc microfibers (3D)	mTeSR1	14 days	96 (2012)
iPSCs	Pronectin F	ReproFF	45 passages	48 (2012)
Chimera protein-immobilized surface				
hESC (H1, H9), hiPSC (hiPSC3a, hiPSC6a)	E-cadherin chimera	mTeSR1	35-53 passages	76 (2010)
hESCs	StemAdhere (chimera E-cadherin)-coating dishes (2D)	TeSR2	not specified	79 (2012)

(*Continiued*)

Table 6.2 Cont.

Cell Lines	Cell Culture Dubstrates (2D or 3D Culture)	Culture Medium	Longest Time in Culture	Ref (Year)
GAG scaffold				
hESCs (HUES7, BG01V/hOG), hiPSCs (PD-iPS5, hFib2-iPS4)	Microfiber of chitin/alginate	mTeSR1	10 passages	81 (2012)
Synthetic polymer surface				
hESCs (H9, BG01)	PMEDSAH surface	StemPro	3-10 passages	85 (2010)
hESCs (HUES1, HUES9)	PMVE-alt-MA surface	StemPro	5 passages	88 (2010)
hESCs (H7, H9, BG01, CHB-8, CHB-10)	PMEDSAH surface	StemPro	10 passages	23 (2011)
hESCs (H1, H9)	APMAAm surface	mTeSR1	20 passages	87 (2011)
hESCs (BG01, H9)	PMEDSAH surface	mTeSR1, StemPro	25 passages	86 (2012)
hESC (RH1)	AEtMA-Cl/DEAEA	mTeSR1	20 passages	89 (2013)

[a]APMAAm, aminopropylmethacrylamide; BSP, bone sialoprotein; GAG, glycosaminoglycan; PMEDSAH, poly[2-(methacryloyloxy(ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide; PMVE-alt-MA, poly(methyl vinyl ether-alt-maleic anhydride); VN, vitronectin

Currently, together with a chemically defined culture medium, cell culture matrices have been investigated to culture hPSCs and to maintain their pluripotency. These matrices are chemically defined and/or void of xenogenic components (Tables 6.1 and 6.2). However, the newly designed matrices require a combination of specific culture media, and only specific hPSCs can maintain pluripotency on these cell culture matrices [31]. Therefore, it is currently difficult to determine the optimal matrices for hPSC culture.

Medical engineering scientists and molecular biologists have recently examined the effects of culture conditions on stem cell fate. Additionally, since 2010, several novel biomaterials and methods for hPSC culture on these biomaterials have been developed under feeder-free and xeno-free culture conditions. Therefore, this chapter mainly focuses on the current developments in hPSC culture materials and describes the biomaterial-assisted regulation of hPSCs under feeder-free and xeno-free culture conditions.

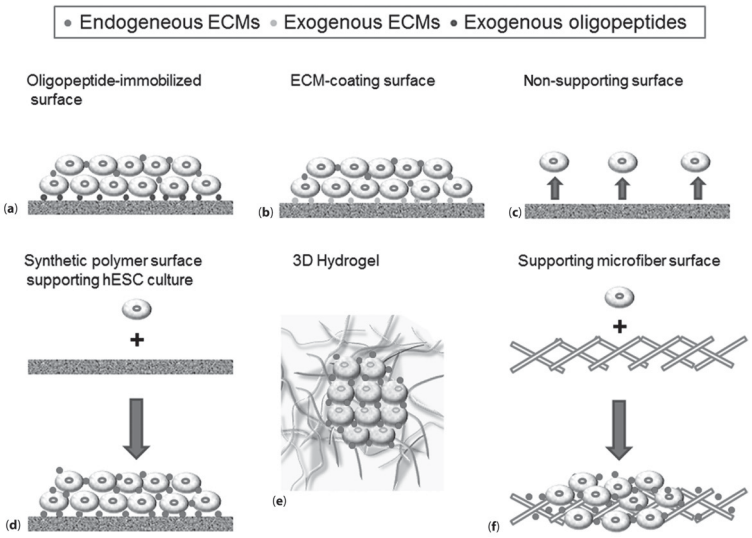


Figure 6.2 Schematic representation and conceptual model of hPSC culture. The hPSC culture on (a) an oligopeptide-immobilized surface (2D and 3D), (b) an ECM-immobilized surface (2D and 3D), (c) a surface that does not support hPSC culture (2D and 3D), (d) a synthetic polymer surface that supports hPSC culture (2D and 3D), (e) a 3D hydrogel prepared with GAG or another polymer, and (f) a microfiber surface that supports hPSC culture. On the oligopeptide-immobilized (a) and ECM-coated substrates (b), which contain exogenous oligopeptides and ECMs, respectively, hESCs can attach via cell-oligopeptide or cell-ECM contacts. Alternatively, hESCs can further produce endogenous ECMs to form extensive cell-matrix contacts and colonies. On synthetic (d,f) and hydrogel (e) substrates that lack exogenous ECMs, hESCs produce ECM (endogenous ECM, red) and establish proximal cell-cell contacts instead of forming extensive cell-matrix contacts and colonies. Reproduced with permission from [2]; Copyright 2014 Elsevier Inc.

There are several strategies for developing biomaterials for hPSC culture under chemically defined and feeder-free conditions. These strategies include hPSC culture (a) on two-dimensional (2D) dishes with immobilized natural extracellular matrix (ECM) polymers, (b) on 2D dishes with immobilized synthetic oligopeptides derived from ECMs, (c) on 2D hydrogels prepared from polysaccharide such as glycosaminoglycan (GAG), (d) on 2D dishes prepared from synthetic monomers, (e) on 3D microcarriers with or without bioactive molecules (ECM, oligopeptides, or GAG) immobilization, and (f) in porous or hydrophilic 3D microcapsules (Figures 6.1 and 6.2).

6.2 Analysis of the Pluripotency of hPSCs

The hPSCs display high telomerase activity and express several pluripotency surface markers, such as glycolipid stage-specific embryonic antigen 4 (SSEA-4), tumor rejection antigen 1-60 (Tra-1-60), keratan sulfate-related antigen, and tumor rejection antigen 1-81 (Tra-1-81), but not glycolipid stage-specific embryonic antigen 1 (SSEA-1). SSEA-1 is expressed on mouse ESCs. High expression levels of specific pluripotency genes are also shown by hESCs, such as Oct3/4, Oct-4 (POU5F1, POU domain

Table 6.3 Characterization of Pluripotent ESCs and iPSCs.[a] Reproduced with permission from [1]. Copyright 2011 American Chemical Society.

Characterization	Specification (Examples)
1. Morphology Cell morphology	Colony formation
2. Protein level Surface marker analysis Immunohistochemical analysis	**Oct-4, Oct-3/4, Nanog, TRA-1-60, Tra-1-81, SSEA-3, SSEA-4** **Oct3/4, Oct-4**, **Sox-2**, **SSEA-3**, **SSEA-4**, **TRA-1-60**, **TRA-1-81, Nanog** **Alkakine phosphatase(AP)** **SSEA-1**(Negative staining)(G77,G207,A1)
3. Gene level	**Oct3/4, Oct-4**, **Sox-2,** Nanog, TDGF-1, UTF-1, REX1, hTERT, ABCG2, DPPA5, CRIPTO, FOXD3, Tert1, Rex2, DPPA5
4. Differentiation ability	**Embryonic body formation in vitro (EB)** **Teratoma formation in vivo**

[a]Bold gene and proteins are frequently analyzed for the characterization of hESCs and human iPSs.

transcription factor), Nanog, Sox-2, Rex-1, and hTERT, the catalytic component of telomerase. Table 6.3 summarizes the characteristics of pluripotent hESCs [1].

The pluripotency of hPSCs is evaluated based on (a) the colony formation analyzed by microscopy, as hPSCs generate colonies with tight boundaries; (b) the alkali phosphatase activity (ALP) measured by colorimetry detection with enzyme-substrate detection; (c) the expression of pluripotency genes evaluated by RT-PCR and qRT-PCR measurements; (d) the expression of pluripotent proteins measured by flow cytometry and immunostaining analysis; and (e) the ability to differentiate into three germ layer cells measured by embryoid body (EB) formation and by teratoma formation, which are analyzed by hematoxylin and eosin (H&E) staining and immunostaining methods (Figure 6.3 [1,2]). It is also important to confirm that the hPSCs do not have any karyotype abnormality after long-term culture.

6.3 Physical Cues of Biomaterials that Guide Maintenance of PSC Pluripotency

Biological cues, such as growth factors, hormones, ECMs, and small chemicals, can guide the pluripotency and differentiation direction of stem cell fate [32,33]. For many years, researchers have devoted substantial effort to identify the soluble factors that mimic the stem cell microenvironment. However, investigators have recently realized the potential importance of the physical cues of biomaterials that influence stem cells;

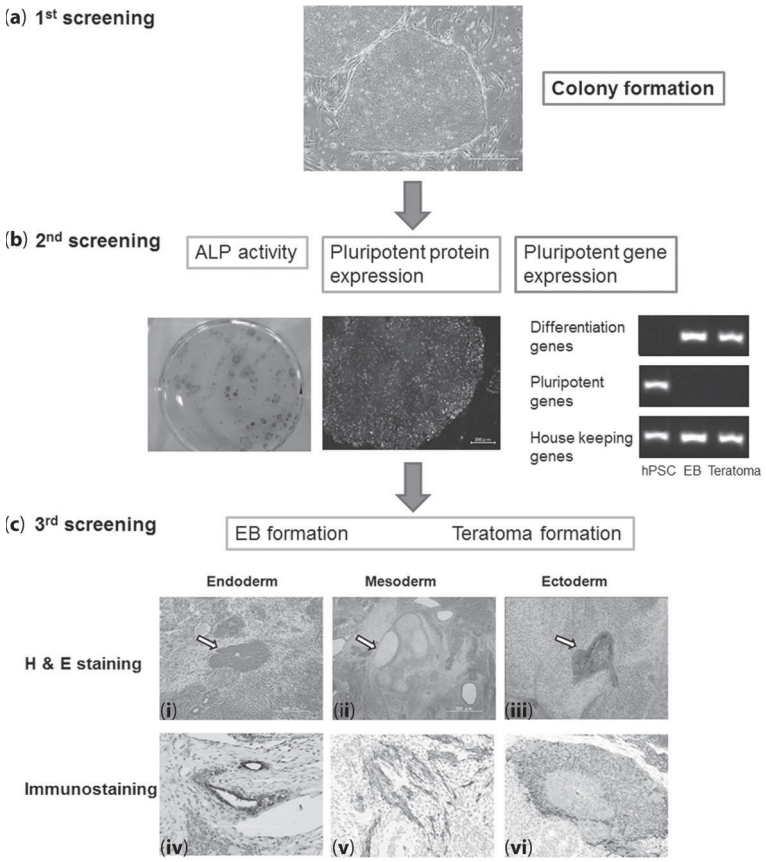


Figure 6.3 Characterization of pluripotent hPSCs. The pluripotency of hPSCs is evaluated based on (a) the colony morphology measured using microscopy (1st screening); (b) the ALP activity measured using an enzyme-substrate reaction, the pluripotent protein expression measured using immunohistochemical or flow cytometry analysis, and the pluripotent gene expression measured using RT-PCR or qRT-PCR analysis (2nd screening); and (c) the ability to differentiate into cells from all three germ layers analyzed using EB and teratoma formation. The pictures in this figure are used with permission from [1]; Copyright 2011, Adapted with permission from American Chemical Society. Reproduced with permission from [2]; Copyright 2014 Elsevier Inc.

these cues include elasticity (stiffness), surface roughness, and the hydrophilicity of the biomaterials used for cell culture [34,35].

Physical interactions between stem cells and the elasticity (or stiffness) of the ECMs where the cells are cultured can guide the stem cell fate of differentiation, although the control of stem cell fate has been classically attributed to genetic and/or epigenetic mediators [36]. Recently, investigators have begun to recognize that the elasticity of cell culture biomaterials guides the lineage commitment of human stem cells. Stem cells tend to efficiently differentiate into specific lineages of tissue cells when the stem cells are cultured on biomaterials with elasticity similar to those tissues [34,35]. Figure 6.4 provides examples of the elasticity of human tissues and natural and synthetic polymers [35].

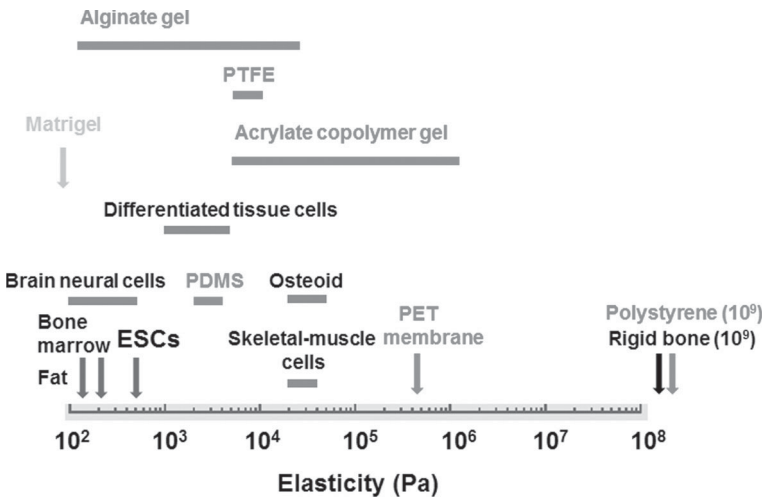


Figure 6.4 Examples of the elasticity of human tissues (blue bars and arrows), synthetic polymers (red bars and arrows), and natural polymers (green arrows). Reproduced with permission from [35]; Copyright 2013 American Chemical Society.

6.3.1 Effect of Biomaterial Elasticity on hPSC Culture

The elasticity of cell culture substrates affects the cell morphology, cell phenotype, and focal adhesions of stem cells, especially in 2D cultures [35]. The mechano-sensing of substrates by stem cells is generated by integrin-mediated focal adhesion signaling [37]. The attachment between cells and the ECM in cell culture substrates or tissues is mediated by integrin receptors. The integrins are composed of obligate heterodimers containing two distinct chains of α and β subunits, which contribute to cell-matrix signaling by activating intracellular tyrosine kinase and phosphatase signaling to elicit downstream biochemical signals that are important for the regulation of gene expression and stem cell fate [38].

Mouse ESCs (mESCs) were shown to maintain their pluripotency on soft substrates (0.6 kPa) whose intrinsic stiffness matched that of the mESCs even in the absence of exogenous leukemia inhibitory factor (LIF). In contrast, mESCs could not maintain their self-renewal and pluripotency on conventional stiff culture polystyrene dishes (> 4 MPa) coated with collagen type I or on hydrogels with much stiffer moduli [39]. In general, LIF had to be added to the culture medium during the expansion of mESCs to maintain their pluripotency and self-renewal [24]. Several mESC cell lines could be cultured on soft substrates without the addition of LIF to the culture medium; these cultures maintained the generation of homogenous undifferentiated colonies with a high expression of pluripotent markers (Oct3/4) and high alkaline phosphatase (ALP) activity (index of pluripotency, see Table 6.3) for up to 15 passages, suggesting that their soft hydrogels could be used for the long-term culture of mESCs [39]. In general, laminin and vitronectin should be more optimal proteins than collagen, which was used in a study that immobilized an ECM in the cell culture substrate to maintain the self-renewal and pluripotency of ESCs and iPSCs [1]. Therefore, it is interesting to note

that mESCs can be cultured on soft hydrogels coated with collagen type I while retaining their self-renewal and pluripotency for 15 passages in the absence of LIF in the culture medium [39]. The mESC colonies on soft cell substrates in a culture medium without LIF generated low cell-matrix traction and had low stiffness. Both the traction and stiffness of the colonies were triggered to increase as the cell culture substrate stiffness increased; these increases were also accompanied by a downregulated expression of the pluripotent protein Oct3/4. This research implies that the self-renewal and pluripotency of mESCs can be maintained on soft cell culture substrates via the biophysical mechanism of facilitating the generation of low cell-matrix traction [39]. Therefore, it appears that an optimal biomaterial elasticity exists for hPSC cultures that maintain the pluripotency of hPSCs [2].

The hESCs (H9) were cultured by our group in a chemically defined medium of mTeSR1 on several grades of stiffness of hydrogels grafted with specific nanosegments [2]. The hESCs cultured on relatively stiffer substrates (e.g., storage moduli of 25 kPa and 30 kPa) tended to differentiate after five days of culture, whereas the hESCs cultured on relatively softer substrates of 12–16 kPa maintained their pluripotency based on the morphology of hESC colony (Figure 6.5) [2]. Only a few small or no colonies of hESCs were observed on the softest substrates (10 kPa). Therefore, our results indicate that cell culture substrates with the optimal elasticity can maintain the pluripotency of a hESC culture [2].

A contradictory result was reported, in which stiff (rigid) substrates can maintain hESC pluripotency [40]. Microfabricated elastomeric PDMS micropost arrays were prepared by Sun *et al.*, and the height of the PDMS microposts controlled the substrate stiffness (elasticity) (Figure 6.6) [35,40]. The PDMS micropost arrays affect focal adhesion, cytoskeleton contractility, cell morphology, and stem cell differentiation [35,40]. hESCs were cultured on oxygen plasma-treated micropost arrays coated with vitronectin. The hESCs were mechano-sensitive, and their cytoskeleton contractility increased with the increasing matrix stiffness of the micropost arrays. Stiff substrates supported the maintenance of the hESC pluripotency [35,40]. Matrix mechanics-mediated cytoskeleton contractility is essentially correlated with E-cadherin expression in cell-cell contacts and is involved in hESC cell fate decisions. The microenvironment of the hESC culture on the micropost arrays significantly differed from that of a conventional 2D culture [35,40]. This different microenvironment may lead to differences in the optimal elasticities (stiffness) required to maintain hESC pluripotency, for which soft surfaces are preferable in a conventional 2D culture and stiff surfaces are preferable in a micropost array culture [2].

6.3.2 Effect of Biomaterial Hydrophilicity on hPSC Culture

Biomaterial wettability can influence the bioresponse of cultured cells [41,42]. Therefore, Li *et al.* prepared vitronectin-coated dishes with different surface charges and wettabilities for hESC culture: vitronectin (VN)-coated tissue culture polystyrene dishes (VN-TCPS), VN-coated polyethylene terephthalate films (VN-coated PET), VN-coated polystyrene films that were plasma-treated in advance (VN-coated PS-plasma), VN-coated PET films that were coated with poly-L-lysine in advance (VN-coated PET+PLL), and VN-coated PET films that were coated with polyacrylic

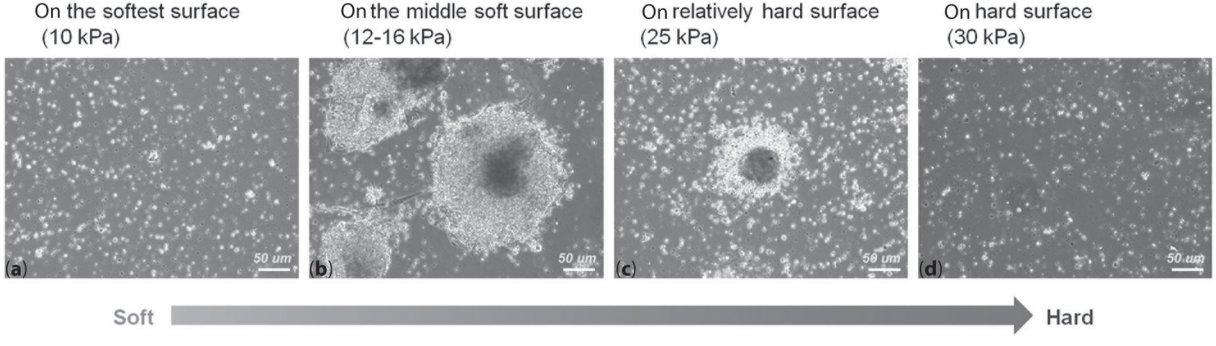


Figure 6.5 H9 hESC culture on hydrogels grafted with specific nanosegments after a five-day culture. (a) hESC morphologies on the soft hydrogel with a 10 kPa storage modulus, (b) hESC morphologies on the middle soft hydrogel with a 12–16 kPa storage modulus, (c) hESC morphologies on a relatively hard hydrogel with a 25 kPa storage modulus, and (d) hESC morphologies on a hard hydrogel with a 30 kPa storage modulus. Reproduced with permission from [2]; Copyright 2014 Elsevier Inc.

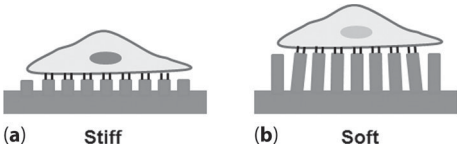


Figure 6.6 Elastomeric polydimethylsiloxane (PDMS) micropost arrays in which the height of the PDMS microposts controls the substrate stiffness (elasticity). Short and long microposts lead to stiff and soft surfaces, respectively, although both micropost arrays are prepared with the same components and PDMS crosslinking. Reproduced with permission from [35]; Copyright 2013 American Chemical Society.

acid in advance (VN-coated PET+PAA) [41]. The VN-coated PET and VN-coated PS-plasma films exhibited greater wettability but did not support the pluripotency of HES-3 hESCs in MEF-CM. MEF-CM is a xeno-containing conditioned medium prepared from mouse embryonic fibroblasts (MEFs). The hESCs cultured on the cationic VN-coated PET+PLL film and the anionic VN-coated PET+PAA film exhibited slower expansion rates than those of the hESCs cultured on the other film types but maintained pluripotency for 13 passages. The highest expansion rates and high pluripotent marker expression were observed in the hESCs cultured on the VN-TCPS dishes, which exhibited a water contact angle of 65 degrees; the data obtained under these conditions were comparable to those for cells cultured on Matrigel [41]. Mei *et al.* also reported that the optimal wettability of culture dishes was an important factor for culturing hPSCs and suggested that a polymer film with a water contact angle of approximately 75 degrees supports hPSC expansion and pluripotency [42].

Mei *et al.* used a combinatorial technique to evaluate biomaterials for culturing hPSCs and maintaining their pluripotency using microarrays [1,2,42]. Twenty-two acrylate monomers with various hydrophobicity and hydrophilicity values (water contact angles) and various crosslinking densities were chosen for development in the microarrays. The colony-formation frequency was investigated by examining the number of polymer spots on which hESC colonies expressed the pluripotent markers Oct-4 and SSEA-4 [42]. The surface roughness of the substrate after fetal bovine serum (FBS) adsorption did not correlate strongly with the colony formation frequency, whereas the optimum wettability ($65° < \text{water contact angle} < 80°$) of the copolymer was well correlated with a high colony formation frequency over a broad range of polymer stiffness values. The polymers with a moderate water contact angle ($65° < \text{water contact angle} < 80°$) that were generated from multiple acrylate-group-containing monomers led to the best colony-formation frequency [1,2,42]. The ability of the hit arrays to expand pluripotent hESCs in defined mTeSR1 medium was verified. These hESCs could differentiate into the cells from three germ layer lineages after more than two months of culture (>10 passages) [1,2,42]. However, in this case, the hESC culture was accomplished on synthetic copolymers with an FBS coating, which indicates that acrylate copolymers alone cannot support hESCs under xeno-free conditions [2].

6.4 Two-Dimensional (2D) Culture of hPSCs on Biomaterials

6.4.1 hPSC Culture on ECM-Immobilized Surfaces in 2D

In addition to MEFs, several human cell lines have been shown to be suitable feeders for hPSC culture. It is rational to assume that adhesion-based cultures of hPSCs rely on the natural ECM polymers deposited by the feeder cells for attachment and proliferation. On the basis of this idea, researchers began to use the ECM components secreted by feeder cells. There are two types of ECMs that are employed for hPSC culture: decellularized ECMs prepared from human fibroblasts from foreskin, dermal, or other human cell lines (which are chemically undefined but not xenogenic) [28,43–46] and single or mixed components of chemically defined ECMs, such as fibronectin, laminin, and vitronectin [47]. These ECMs are used as coating materials on cell culture dishes or scaffolds [48].

Fu *et al.* prepared ECMs derived from decellularized feeders that had been derived from embryonic bodies that were predifferentiated from H9 hESCs through a freeze-thaw procedure (Table 6.1) [43]. ECMs composed of collagen IV, laminin, and fibronectin were used to support the long-term growth of H1 and H9 hESCs with animal component-free TeSR2 medium for up to 20 passages [43]. Therefore, this study suggested a new strategy for the efficient propagation of clinically compliant hESCs in an autologous, feeder-free culture system.

Most of the reported hPSC lines were initially derived via culture on MEFs during their initial development. Therefore, these cell lines are difficult to use for the clinical treatment of human patients. Ilic *et al.* derived and propagated hESCs on decellularized human neonatal foreskin fibroblasts under feeder-free and xeno-free conditions (Table 6.1) [44]. Their specific mutation-carrying (Huntington's disease and myotonic dystrophy 1) hESC lines maintained pluripotency on these decellularized ECMs. However, dishes coated with commercially available CELLstart (mixture of fibronectin and human serum albumin) did not support the growth of these hESCs in serum-free medium (StemPro) containing bFGF, insulin growth factor 1 (IGF1), heregulin, and activin A [49]. In contrast, conventional hESC lines can generally maintain pluripotency on CELLstart in the StemPro medium [44]. Currently, the combination of a specific cell culture matrix and culture medium must be carefully selected and fine-tuned depending on the specific characteristics of particular hPSCs [31]. There is no perfect coating material or cell culture dish that can ensure the maintenance of hPSC pluripotency for a long period of time (i.e., over 10–20 passages).

Natural polymers of recombinant or natural fibronectin, laminin, vitronectin, and collagen IV, which are components of ECMs, have begun to be used instead of Matrigel or decellularized ECMs for the feeder-free growth of undifferentiated hPSCs because their chemical characteristics are relatively more well defined (Table 6.1). The ECM components that are immobilized on dishes for hPSC proliferation and to provide binding sites for stem cells are summarized in Table 6.4 [50].

Table 6.4 ECMs immobilized on dishes for adhesion, differentiation, and proliferation of stem cells and binding site of cells. Reproduced with permission from [50]. Copyright 2012 American Chemical Society.

ECM	Binding Site of Cells
Collagen I	Integrin (αVβ3, α2β1)
Collagen I	Integrin (α1β1)
Collagen I	Integrin (α1β1, α2β1, α3β1)
Collagen II	Integrin (α1β1, α2β1, α10β1)
Collagen IV	Integrin (α2β1, CD44)
Fibronectin	Integrin (α4β1, α5β1, αVβ3, αIIbβ3, αVβ6, αVβ5)
Laminin	Integrin (α1β1, α2β1, α3β1, α6β1, α6β4)
Laminin-1 (laminin 111)	Integrin (α1β1, α2β1, α6β1, α7β1, α9β1), α-dystroglycan, suifade, and heparan sulfate proteoglycan
Laminin-5 (laminin 332)	Integrin (α2β1, α3β1, α6β1, α6β4)
Laminin-511	Integrin (α6β1)
Laminin-10/11	Integrin (α3β1, α6β1, α6β4)
Vitronectin	Integrin (αVβ3, αVβ5)

6.4.1.1 Fibronectin

Fibronectin is a high-molecular-weight (~440 kDa) glycoprotein component of ECMs that binds to other ECM components, such as collagen, fibrin, and heparan sulfate proteoglycans. Fibronectin exists as a protein dimer consisting of two nearly identical monomers linked by a pair of disulfide bonds [2].

Several investigators have evaluated that fibronectin-coated substrates maintain hESC pluripotency (Table 6.1) [51–58], whereas other researchers have reported unfavorable results when culturing hESCs on fibronectin-coated substrates [31,59]. Amit *et al.* cultured several hESC lines (I-6, I-3, and H-9) on bovine and human fibronectin-coated dishes (5 μg/cm^2) in knockout DMEM (KO-DMEM) supplemented with 15% serum replacement (SR). The fibronectin-specific integrin receptor α5β1 was expressed in the undifferentiated hESCs (Figure 6.7) [52]. Under these conditions, the hESCs maintained pluripotency for more than six months, whereas the hESCs cultured on gelatin-coated dishes tended to differentiate [52]. Human fibronectin was found to be relatively more favorable for maintaining the pluripotency of the hESCs compared with bovine fibronectin [52]. The applied SR contained Albumax, which is a lipid-enriched bovine serum. Therefore, this work was not performed under xeno-free culture conditions.

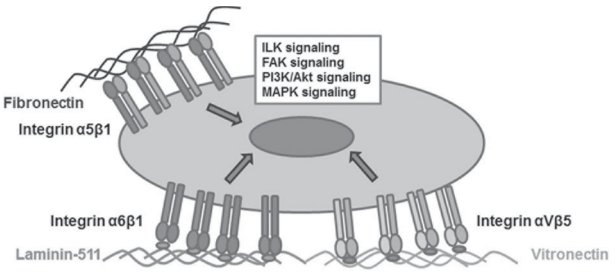


Figure 6.7 Schematic model of hESC adhesion on an ECM-coated substrate. The adhesion of hESCs to fibronectin, vitronectin, and laminin-511 is mediated by $\alpha_5\beta_1$, $\alpha_V\beta_5$, and $\alpha_6\beta_1$, respectively. Reproduced with permission from [2]; Copyright 2014 Elsevier Inc.

Hughes *et al.* analyzed commercially available cell culture matrices for hPSCs, including CELLstart (Invitrogen), StemXVivo (R&D Systems), and Bridge Human ECM (Global Stem), as well as human basement extract and Matrigel (Table 6.1) [53]. StemXVivo is marketed as a mixture of recombinant proteins and is primarily composed of enactin and vitronectin. The main components of CELLstart are human fibronectin and human serum albumin [53]. Bridge Human ECM is primarily composed of albumin and also contains fibronectin isoform-1. Fibronectin is a highly abundant component of these matrices. Hughes *et al.* found that hESCs (H9 and CA1 lines) could maintain their pluripotency and their capacity for self-renewal on fibronectin-coated dishes as well as on commercially available composite ECMs, including fibronectin in defined mTeSR1 medium containing transferrin, IGF, bFGF, TGF-β1, and bovine serum albumin (BSA). However, none of these matrices could promote and maintain the growth of H9 hESCs with the same efficiency and stability as Matrigel in defined mTeSR1 medium [53]. The authors suggested that fibronectin, either with or without other defined ECM components, had the ability to maintain the pluripotency and undifferentiated state of H9 and CA1 hESCs in mTeSR1 to some extent [53]. However, fibronectin alone does not appear to represent a perfect matrix for culturing pluripotent hESCs based on these results. Many researchers are using CELLstart as an hPSC adhesion matrix *in vitro* [51,54,55,60]. Hernandez *et al.* cultured hESCs (Shef3 and Shef6) on CELLstart in StemPro medium, which maintained their pluripotency and normal karyotype for over 20 passages (Table 6.1) [51]. These authors also succeeded in propagating hESCs from a single cell to clonal expansion [51]. The clonal expansion of hESCs is advantageous for the safe selection of hESCs or hiPSCs for clinical applications, especially after genetic modification of the cells.

Sugii *et al.* reported a feeder-free production of hiPSCs from human adipose-derived stem cells (hADSCs) on the humanized, defined substrate CELLstart (Table 6.1) [54,55]. The hiPSCs were successively generated on the substrate, although more time was required to generate hiPSC colonies via feeder-free production methods than for feeder-dependent methods [55]. Hayashi *et al.* also examined hiPSC generation from adult human dermal fibroblasts under feeder-free and defined culture conditions (Table 6.1) [61]. The hiPSCs were cultured on fibronectin-coated dishes in an hESF9a-based medium. Because of the use of a defined medium and matrix, the established

hiPSC lines showed little or no expression of non-human N-glycolylneuraminic acid (Neu5Gc), which causes immunorejection when the hiPSCs are transplanted into human patients [61]. Their results show that feeder-free iPSCs can be produced using fibronectin-coated dishes.

6.4.1.2 Laminin

Laminin is the first ECM protein expressed in two- to four-cell-stage embryos and is a major component of the ECM of the basal laminae in vertebrates [1]. Laminins are a family of heterotrimeric glycoproteins composed of α, β, and γ chains, forming 15 different combinations in human tissues [62]. Laminin-511 consists of the α5, β1, and γ1 chains and is frequently employed as a coating material on the dishes used to culture hESCs (Table 6.1) [19,62–65].

Several hESC lines express laminin-511 chains, whereas laminin-332 is not expressed in hESCs [62,63,66]. Miyazaki *et al.* found that hESCs primarily express integrin α6β1, which binds predominantly to laminin-111, laminin-332, and laminin-511/-521 (Figure 6.7) [63]. The hESCs were observed to proliferate on plates coated with these three recombinant laminins in MEF-CM for several passages while maintaining their pluripotency. These results showed that recombinant laminin-111, laminin-332, and laminin-511 can aid in the expansion of undifferentiated hESCs due to their high affinity for integrin α6β1, which is expressed on hESCs [63]. However, this work was performed using xeno-containing culture media, and the hESCs were only cultured for a few passages. It is therefore unknown whether the hESC pluripotency can be maintained for extended passages in xeno-free culture medium. However, Rodin *et al.* reported that hESCs (HS420, HS207, and HS401 lines) cultured on laminin-511-coated dishes could maintain pluripotency for at least 20 passages with a normal karyotype in either O3 medium or H3 medium, which are composed of animal derivative-free components [62].

6.4.1.3 Vitronectin

Vitronectin is a 75 kDa glycoprotein consisting of 459 amino acid residues [67]. Braam *et al.* studied hESC attachment on dishes coated with several ECMs (laminin-111, vitronectin, fibronectin, and collagen IV) at concentrations of 10–50 μg/ml (Table 6.1) [59]. Integrin-blocking experiments suggested that αVβ5 integrins mediated the adhesion of hESCs to vitronectin, α5β1 mediated the adhesion to fibronectin, and α6β1 mediated the adhesion to laminin-111 as well as laminin-332 and laminin-511 (Figure 6.7) [59,63]. Only the recombinant vitronectin-coated dishes supported the self-renewal and pluripotency of hESCs for at least eight passages in defined mTeSR1 medium in this study. Several other researchers have also reported that vitronectin-coated dishes support the pluripotency of hESCs (Table 6.1) [31,41,68–70]. Yap *et al.* defined a threshold surface density of vitronectin for the expansion of hESCs (H1 and HES-3) and for the maintenance of pluripotency [68]. The successful propagation of hESCs was found to be possible for > 20 passages on dishes coated with > 5 μg/ml human plasma vitronectin in mTeSR1 medium, which corresponded to 250 ng/cm^2 of protein as determined by the Bradford assay [68].

Meng *et al.* investigated the synergistic effects of the cell culture medium, matrix, and exogenous factors on the adhesion and growth of hPSCs under defined and xeno-free culture conditions in which hPSCs maintain pluripotency for a long period of time [31]. These authors found that hPSC culture on vitronectin-coated dishes in defined TeSR2 medium was optimal for maintaining long-term cultures of three hESC lines and two hiPSC lines, whereas hPSC culture on fibronectin-coated dishes was suboptimal [31]. Vitronectin, in concert with a suitable cell culture medium, can be regarded as one of the optimum matrices for supporting hPSC proliferation.

6.4.2 hPSC Culture on Oligopeptide-Immobilized Surfaces in 2D

Culturing hESCs on ECM components, such as vitronectin and laminin-511, which are used to coat dishes, maintains hESC self-renewal and the ability of the cells to differentiate into the three germ layer lineages for a long time period in a defined culture medium. However, these ECMs are costly and are derived from animal sources, leading to batch-to-batch variability. It is desirable to use synthetic oligopeptide materials as functional and active sites to maintain the pluripotency of hPSCs. Melkoumian *et al.* developed synthetic surfaces composed of polyacrylate conjugated to biologically active peptides (PAS) using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide (EDC/NHS) reaction [71]. These biologically active peptides on PAS were bone sialoprotein oligopeptide (BSP-PAS), vitronectin oligopeptide (VN-PAS), long or short fibronectin oligopeptide (sFN-PAS or lFN-PAS, respectively), and laminin oligopeptide (LM-PAS) (Tables 6.2 and 6.5) [71]. Only the BSP- and VN-derived peptides generated hESC (H1 and H7 lines) adhesion and colony formation; this study was the first to demonstrate hESC pluripotency when hESCs were cultured on oligopeptide-immobilized dishes in defined X-VIVO medium. Interestingly, all of the oligopeptides investigated in this study contained the RGD (arginine-glycine-aspartic acid) cell adhesion sequence, suggesting that the RGD sequence alone is not sufficient for adherence and the maintenance of hESC self-renewal and pluripotency [71]. The surface density of the oligopeptide was also found to be important, with a BSP peptide concentration of > 0.5 mM being necessary to maintain the hESC colony on the peptide surfaces [71]. This concentration is an extremely high concentration. The hESCs (H7) were cultured on BSP-PAS and VN-PAS surfaces for more than ten serial passages and maintained self-renewal with a normal karyotype in defined media, including X-VIVO 10, mTeSR1, and TeSR2 [71].

The cyclic RGD peptide CRGDC is known to have ten-fold greater ability than the linear GRGDSP peptide to bind the αVβ3, αVβ5, and α5β1 integrins where the αVβ5 and α5β1 integrins are expressed in hESCs [59,72]. Therefore, Kolhar *et al.* designed cyclic RGD peptide-grafted dishes. GACRGDCLGA peptides were conjugated to amine-modified tissue culture plates via a bi-functional linker, which reacts with an amine on the culture substrate and a thiol of the peptide (Figure 6.8) [73]. The cyclic RGD peptide density on the plates was calculated to be 10–30 fmol of peptide per cm^2. The cyclic RGD peptide-grafted dishes supported the expansion of hESCs (H9 and H14) with the maintenance of pluripotency in MEF-CM for ten passages [73]. The differentiation ability of hESCs into three germ layers after ten passages was confirmed by

Table 6.5 Amino acid sequences of oligopeptides and polypeptides for hPSC culture.[a] Reproduced with permission from [2]. Copyright 2014 Elsevier Inc.

Name (Model ECM)	Amino Acid Sequence	Ref (Year)
BSP-PAS (BSP)	KGGNGEP**RGD**TYRAY	71 (2010)
VN-PAS (VN)	KGGPQVT**RGD**VFTMP	71 (2010)
sFN-PAS (FN)	G**RGD**SPK	71 (2010)
lFN-PAS (FN)	KGGAVTG**RGD**SPASS	71 (2010)
LN-PAS (LN) Cyclic RGD	KYGAAS**IKVAV**SADR GAC**RGD**CLGA	71 (2010) 73 (2010)
Oligo-HBP1 (VN)	GKKQRFRHRNRKG	74 (2012)
Oligo-HBP2 (BSP)	FHRRIKA	74 (2010)
Oligo-HBP3 (FN)	GWQPPARARI	74 (2010)
PDL	-(-Lysine-)-$_n$	31 (2012)
Pronectin F (FN)	([GAGAGS]$_9$GAAVT**RGD**SPASAAGY)$_{12}$	48 (2012)

[a]FN, fibronectin; BSP, bone sialoprotain, HBP, heparin binding peptides; LN, laminin; PDL, poly-D-lysine; VN, vitronectin

EB formation. The hESCs were reported to be cultured in a chemically defined medium, mTeSR1, for five days in this study [73]. Dishes with optimal elasticity that are grafted with cyclic RGD peptide might improve the culture of hESCs in xeno-free and chemically defined medium for long passages (10–20 passages).

Klim *et al.* used a defined-surface array of self-assembled monolayers composed of peptide-substituted alkanethiol conjugates to identify specific peptide sequences that sustain the self-renewal of hPSCs (Table 6.2) [74]. A total of 18 different bioactive oligopeptides were identified; these oligopeptides were derived from fibronectin, laminin, vitronectin, bone sialoprotein, annexin, E-cadherin, NCAM, Dll-1, BMP-2, and phage display libraries. These oligopeptides contained not only the integrin ligand RGD but also the binding domain of polysaccharide (Table 6.5) [74]. The surfaces presenting heparin-binding peptides (GKKQRFRHRNRKG from vitronectin, FHRRIKA from bone sialoprotein, and GWQPPARARI from fibronectin) consistently mediated hESC (H9) adhesion and allowed for hESC propagation associated with pluripotent marker expression in mTeSR1 medium with the ROCK inhibitor Y-27632. In contrast, the integrin ligand KGRGDS inconsistently maintained hESC pluripotency [74]. Several hESC lines (H9, H13, H14, and DF19-97T) and hiPSCs (IMR-90) cultured on dishes with the immobilized GKKQRFRHRNRKG sequence

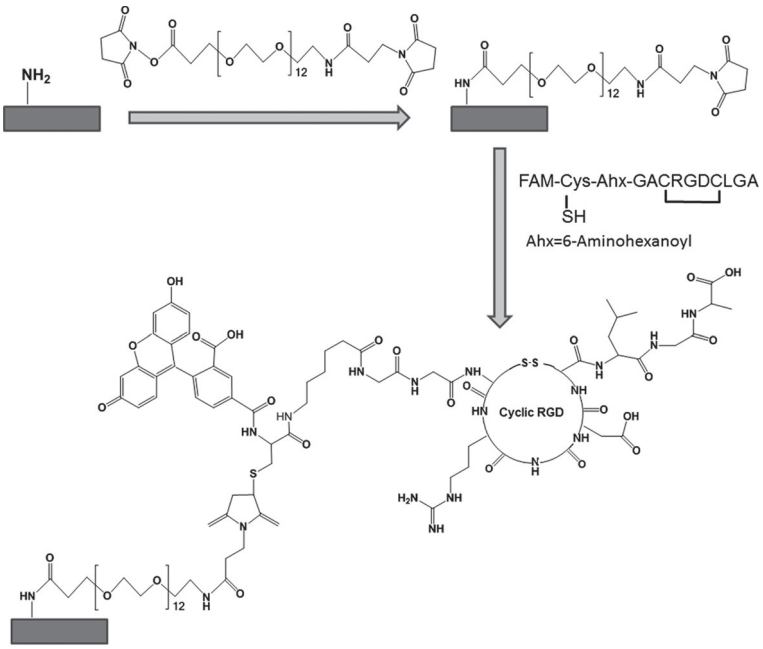


Figure 6.8 Preparation of a cyclic RGD peptide-grafted surface. GACRGDCLGA peptides were conjugated to amine-modified tissue culture plates via a bi-functional linker, which reacts with an amine on the culture substrate and a thiol of the peptide. This figure was adapted with permission from [73]; Copyright 2010 Elsevier Inc.

using biotin-streptavidin chemistry in mTeSR1 together with ROCK inhibitors were found to proliferate and exhibit long-term self-renewal and pluripotency (up to three months, 17 passages) with normal karyotypes. These cells differentiated into three germ layer lineages after three months of culture. The hESCs could also be cultured on dishes with the immobilized GKKQRFRHRNRKG sequence and the cyclic RGD peptide for one to two months in the absence of a ROCK inhibitor in defined mTeSR1 medium, which maintained their self-renewal ability and pluripotency together with normal karyotypes [74]. Specific oligopeptide-immobilized dishes are able to maintain hPSCs in a defined medium and are preferable to ECM-coated dishes because of their fully synthetic nature.

6.4.3 hPSC Culture on Recombinant E-cadherin Substratum in 2D

E-cadherin is a Ca^{2+}-dependent cell-cell adhesion molecule [75] that is essential for intercellular adhesion and colony formation among hESCs [1]. Undifferentiated hESCs express large amounts of E-cadherin. Nagaoka *et al.* prepared a fusion protein consisting of the E-cadherin extracellular domain and the IgG Fc domain (E-cad-Fc) (Figure 6.9) and investigated hESC culture on the recombinant E-cadherin substratum in both MEF-CM and defined mTeSR1 medium (Table 6.2) [1,76]. The hESCs cultured in this manner could maintain pluripotency for > 35 passages and could differentiate into the three germ layer lineages.

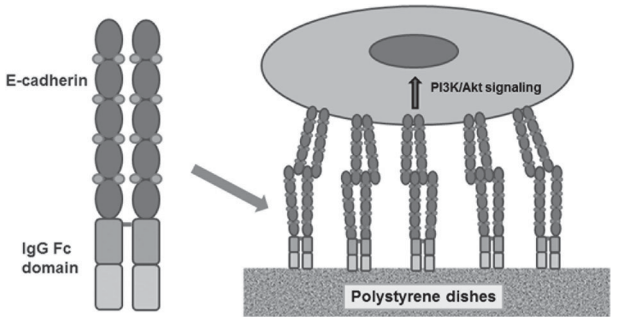


Figure 6.9 Schematic model of hESC adhesion on an E-cadherin chimera-coated surface. The E-cadherin-mediated adhesion of hESCs is typically associated with β-catenin signaling and stimulates PI3K/Akt signaling. Reproduced with permission from [2]; Copyright 2014 Elsevier Inc.

Integrin-mediated cell-ECM interactions are considered essential for the maintenance of stem cell pluripotency and viability. As noted in previous sections, substantial effort has been devoted to finding a suitable ECM component that can maintain the pluripotency of hESCs via the interaction between hESCs and integrin receptors on ECMs. Integrin-ECM interactions activate integrin-linked kinase (ILK) or focal adhesion kinase (FAK) signaling pathways as well as the PI3K/Akt and MAPK pathways [77]. In contrast, the E-cadherin-mediated adhesion of hESCs is typically associated with *β*-catenin signaling and also stimulates PI3K/Akt signaling (Figures 6.7 and 6.9) [76]. The Akt signaling pathways are considered to be especially important for maintaining the pluripotency of hESCs [78]. It has been suggested that trans-homodimerization between E-cadherin on hESCs and the E-cadherin domain of a substratum of recombinant E-cadherin could promote and maintain the pluripotency of hESCs via the activation of the PI3K/Akt signaling pathway [76]. A recombinant E-cadherin substratum is currently commercially available (StemAdhere™ from Stem Cell Technologies) [79].

6.4.4 hPSC Culture on Polysaccharide-Immobilized Surfaces in 2D

Several polysaccharides, such as hyaluronan and alginate, have also been examined as potentially suitable biomaterials for hPSC expansion [80–83]. Polysaccharide is known to play an important role in regulating hPSC adhesion and self-renewal [74]. For example, hyaluronan-derived hydrogels might provide an adequate physiological environment for hPSC growth because the feeder layers that support hPSCs are composed of large amounts of hyaluronic acid in addition to abundant ECM proteins.

Liu *et al.* developed hyaluronan-derived hydrogels from thiol-modified hyaluronan, thiol-modified gelatin, and poly(ethylene glycol) diacrylate both with and without fibronectin [80]. The growth rates of hiPSCs (HDFa-YK26) on the hyaluronan-derived hydrogels (with and without fibronectin) in MEF-CM were similar to those of hiPSCs grown on MEF or Matrigel for 12 serial passages. Additionally, these cells maintained their pluripotency, their ability to differentiate into three germ layer lineages *in vitro,* and their stable karyotypes [80]. Furthermore, hiPSCs could be cultured on

hyaluronan-derived hydrogels containing fibronectin but not on hyaluronan-derived hydrogels without fibronectin in defined NutriStem medium. However, the long-term culture of hiPSCs on hyaluronan-derived hydrogels containing fibronectin in NutriStem medium was not successful due to the mechanical weakness of the hyaluronan-derived hydrogels containing fibronectin [80].

Compared with 2D culture, 3D culture more accurately imitates the *in vivo* microenvironment by allowing extensive cell-cell and cell-matrix contacts and interactions. The problem with most 3D culture systems is that harvesting expanded hPSCs from 3D scaffolds is difficult. Lu *et al.* constructed ion complex microfiber scaffolds composed of anionic alginate and citosan; these polysaccharides can be enzymatically degraded using alginate lysate and chitinase, respectively [81]. hESC-embedded microfibers were prepared by spinning microfibers from the interface between a chitosan solution and an alginate solution containing hPSCs followed by immersion in a $CaCl_2$ solution for alginate crosslinking (Table 6.2, Figure 6.10 [81]). Then, several types of hESCs and hiPSCs were loaded onto the 3D microfiber system, and these cells could be propagated for ten consecutive passages in defined mTeSR1 medium while maintaining their pluripotency and their ability to differentiate into the three germ layer lineages while showing stable karyotypes. The 3D microfibers allowed for the encapsulation of hPSCs at a high seeding density (approximately 10^7 cells/ml) while maintaining high viability. This density was approximately 50 times higher than the cell density achieved with a conventional 2D culture system [81]. hPSC harvesting was accomplished by incubating the 3D microfibers in chitinase and alginate lysate for 10 min. The hPSCs recovered from the 3D microfibers showed excellent cell viability and pluripotency. Furthermore, the hPSCs embedded in 3D microfibers were directly cryopreserved, and more than 75% of these hPSCs were viable after thawing in mTeSR1 containing ROCK inhibitors. This recovery ratio was more than 17-fold higher than that observed in hPSCs cryopreserved using the

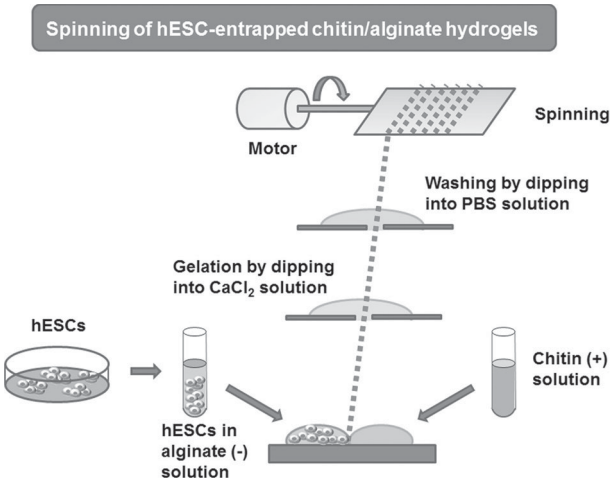


Figure 6.10 Spinning of hESCs entrapped in chitosan/alginate ion complex hydrogels. Microfibers were prepared by spinning from the interface between the chitosan solution and the alginate solution containing hPSCs, followed by immersion in a $CaCl_2$ solution for alginate crosslinking (coagulation) and washing. Figure modified with permission from [81]; Copyright 2012 Elsevier Inc.

conventional method [81] because this 3D microfiber system obviated the need for cell detachment before cryopreservation.

6.4.5 hPSC Culture on Synthetic Surfaces in 2D

The culture of hPSCs on chemically defined substrates synthesized from monomers eliminates the variables associated with feeder cells and natural protein coatings, which can range from batch-to-batch inconsistencies to biosafety issues [23]. In most cases, synthetic polymer matrices support only short-term hPSC propagation or culture in culture media containing xenobiotic products, such as fetal bovine serum (FBS) or MEF-CM [42,84]. Recently, several synthetic polymer materials were reported to support hPSC proliferation in xeno-free conditions or in chemically defined media (Table 6.2 and Figure 6.11) [85–89]. In this section, we will discuss (a) several types of synthetic polymers that sustain the long-term culture of hPSCs, (b) the thermoresponsive synthetic polymers that hold hPSC culture, and (c) synthetic nanofibers that maintain the long-term culture of hPSCs.

6.4.5.1 Synthetic Polymers that Support the Long-Term Culture of hPSCs

A fully defined synthetic polymer, poly(2-[methacryloyloxy]ethyl dimethyl-[3-sulfopropyl] ammonium hydroxide) (PMEDSAH), was grafted onto TCPS dishes via

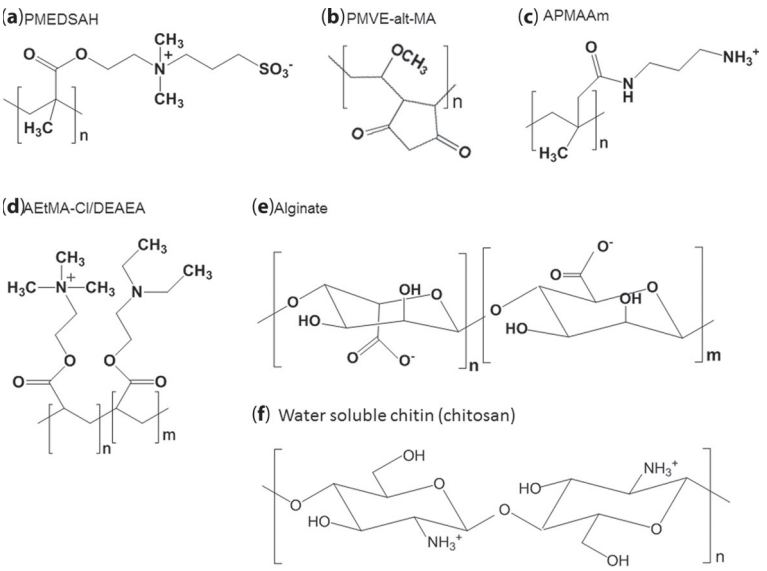
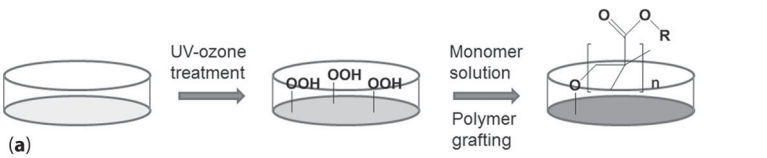
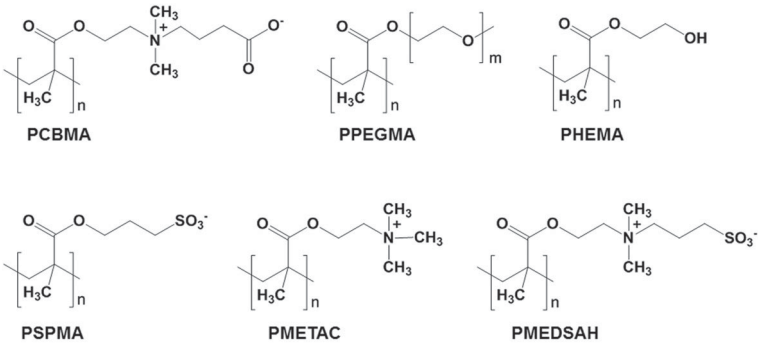


Figure 6.11 Chemical structures of the synthetic polymers used as substrates, hydrogels, and scaffolds for the proliferation of pluripotent hPSCs in defined media under feeder-free and xeno-free conditions. (a) Poly(2-[methacryloyloxy]ethyl dimethyl-[3-sulfopropyl] ammonium hydroxide) (PMEDSAH), (b) poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA), (c) aminopropylmethacrylamide (APMAAm), (d) copolymer of 2-(diethylamino)ethyl acrylate (DEAEA) and 2-(acryloyloxyethyl) trimethylammonium chloride (AEtMA-Cl) (AEtMA-Cl/DEAEA), e) alginate, and (f) water-soluble chitin (chitosan). Reproduced with permission from [2]; Copyright 2014 Elsevier Inc.



(**a**)



(**b**)

Polymer	PCBMA	PPEGMA	PHEMA	PSPMA	PMETAC	PMEDSAH
Contact angle	71.6	63.3	56.0	50.2	40.5	17.1
Reduced elastic modulus (GPa)	3.3	3.9	3.0	3.0	3.3	2.5
Cell-aggregate adhesion (%)	0	5	12	14	8	15
Number of passages	0	1	2	2	2	**25**

(**c**)

Figure 6.12 Long-term culture of H9 hES cells on synthetic dishes in MEF-CM. (a) Schematic diagram showing the graft-polymerization process used to synthesize the polymer grafting. Tissue culture polystyrene dishes were first activated by UV ozone; then, methacrylate-based monomers were polymerized from the surface. (b) Chemical structure of the methacrylate-based monomers used for the polymer grafting on the dishes. (c) The table lists the contact angle, reduced elastic modulus (GPa), initial hES cell aggregate adhesion, and number of passages achieved on each polymer-grafted dish. Figure modified with permission from [86]; Adapted 2012. Reproduced with permission – Creative Commons License.

surface-initiated graft polymerization (Table 6.2 and Figure 6.12 [86]), which supported the long-term culture of hESCs (H9) with normal karyotypes for more than ten passages in defined StemPro medium [85,86]. However, no hESC attachment was observed on poly(carboxybetaine methacrylate) (PCBMA)-grafted dishes, and the hESCs adhered but spontaneously differentiated during the first two passages on poly([2-(methacryloyloxy)ethyl]) (PMETAC)-, poly(3-sulfopropyl methacrylate) (PSPMA)-, poly(2-hydroxyethyl methacrylate) (PHEMA)-, and poly(poly[ethylene glycol] methyl ether methacrylate) (PPEGMA)-grafted dishes in MEF-CM (Figure 6.12 [86]). Only PMEDSAH-grafted dishes successfully supported H9 cells in StemPro medium [85,86]. Some hESC lines, such as BG01, could not maintain pluripotency beyond three passages on the PMEDSAH-coated dishes in StemPro medium, although the development of completely synthetic surfaces, such as a PMEDSAH-grafted dish, is essential for the proliferation and maintenance of pluripotent hESCs in defined and xeno-free culture media.

The hydrogel interface of aminopropylmethacrylamide (APMAAm) was reported to be adequate for culturing hESCs in the defined medium mTeSR1 (Table 6.2, Figure 6.11) [87]. Healy *et al.* reported that hESCs (H1 and H9) could be cultured on APMAAm for over 20 passages and could maintain pluripotency, similar to the results observed for hESCs cultured on Matrigel (both qualitatively and quantitatively) [87]. These authors analyzed the adsorption of BSA and other components, such as bFGF and TGF-β, onto an APMAAm hydrogel in mTeSR1 media and found that the adsorption of certain specific proteins on the surface was critical for hESC adhesion to the hydrogel [87]. Although BSA is known to block the nonspecific adsorption of other proteins on the surfaces, the specific conformation of BSA as well as that of other components of the medium on APMAAm appears to create an interface that supports the long-term growth of hESCs. Albumin, along with other bioactive components, may facilitate the adhesion of hPSCs by promoting the secretion of ECMs from hPSCs and/or integrin expression by hPSCs when albumin is adsorbed on the surfaces with a specific conformation.

Other types of synthetic polymers can also support the proliferation of pluripotent hESCs with normal karyotypes. Brafman *et al.* prepared dishes coated with 90 different synthetic polymers for use in a high-throughput screening approach [88]. Among the tested polymers, poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA), with a molecular weight of approximately 1.25 x 10^6 Da, was observed to support the proliferation and self-renewal of hESCs (HUES1 and HUES9) over five passages in StemPro medium (Table 6.2, Figure 6.11), whereas most of the other polymers supported hESC proliferation for only a short period of time [88]. ECMs are known to be secreted by hESCs and play a critical role in cell attachment and the maintenance of pluripotency, whereas the integrins α5 and αV are primary integrins that mediate hESC attachment and interactions between ECMs. The expression levels of endogenous ECMs and integrins in hESCs cultured on PMVE-alt-MA were found to be significantly higher than their levels in hESCs grown on Matrigel [88]. Therefore, PMVE-alt-MA appears to promote both the secretion of ECMs and the expression of integrins by hESCs, which supports the proliferation of pluripotent hESCs on PMVE-alt-MA-coated dishes (Figure 6.2(d)).

6.4.5.2 Thermoresponsive Synthetic Polymers that Support hPSC Culture

Mechanical, enzymatic, and chemical cell dissociation methods are currently used as passaging techniques for hPSCs. However, these methods are not well defined, which introduces variability into the system and may lead to cell damage. The enzymatic detachment of cells from dishes sometimes generates karyotype abnormalities.

Cells can be detached from substrates by external stimulation, such as a temperature change or light irradiation [90–93]. In particular, the dishes or micelles prepared from a thermoresponsive polymer exhibiting a low critical temperature (LCST) have been used for drug carriers, cell culture dishes, and other biomedical usages [94]. Zhang *et al.* screened thermoresponsive synthetic polymer arrays consisting of 609 different polymers, spotted in quadruplicate, that were synthesized *in situ* via inkjet printing mixtures of 18 monomers in seven different ratios in the presence of the crosslinker *N,N'*-methylene-bis-acrylamide for hESC (RH1) culture [89]. These authors found that a family of thermoresponsive synthetic hydrogels based on 2-(diethylamino)

ethyl acrylate (DEAEA) and 2-(acryloyloxyethyl) trimethylammonium chloride (AEtMA-Cl), AEtMA-Cl/DEAEA, supported the long-term growth and pluripotency of hESCs (RH1 and H9) in defined mTeSR1 medium over a period of two to six months (more than 20 passages) (Figure 6.11, Table 6.2) [89]. The hydrogels permitted gentle, reagent-free cell passaging associated with > 90% cell release through the transient modulation of the ambient temperature from 37 to 15°C for 30 min. The chemically defined substrates developed in this study represent a flexible and scalable approach for improving the definition, efficacy, and safety of hPSC culture systems for research, industrial, and clinical applications [89].

6.4.5.3 Synthetic Microfibrous Scaffolds that Support the Long-Term Culture of hPSCs

Currently, most of the available synthetic polymers that support hPSC proliferation are hydrogels, such as PMEDSAH [85,86], APMAAm [87], PMVE-alt-MA [88], and AEtMA-Cl/DEAEA [89], that are used in conjunction with defined and/or xeno-free media (Table 6.2 and Figure 6.11). The systematic design of hydrogels might facilitate the development of biomaterials for the long-term culture of pluripotent hPSCs. The development of chemically defined, controllable, and sustainable culture matrices composed of synthetic polymer hydrogels on dishes or microcarriers is critical for elucidating the mechanisms that control hPSC cell behavior and for optimizing the conditions for the safe clinical application of hPSCs.

Conventional hPSC culture systems mainly involve 2D dishes, which fail to adequately recapitulate the complex 3D microenvironments that cells inhabit during embryonic development [95]. Therefore, conventional 2D cultures of hPSCs *in vitro* have limited ability to reproduce the 3D *in-vivo* microenvironment. Carlson *et al.* investigated critical determinants of hESC (H1 and H9) self-renewal on poly-D-lysine (PDL)-immobilized synthetic polymer substrates with different configurations, which included 2D polymer films, macroporous scaffolds (sponges) with a nonfibrous geometry, and microfibrous scaffolds prepared via the electrospinning method (Table 6.2). The base polymer was poly(desaminotyrosyl tyrosine ethyl ester carbonate) (pDTEc). The hESCs failed to adhere to both the 2D substrates and the 3D macroporous scaffolds coated with PDL. However, the electrospun microfibers coated with PDL were able to promote cell adhesion, proliferation, and pluripotency and were able to maintain the differentiation ability of hESCs for at least 14 days in defined mTeSR1 medium [96]. However, the long-term culture of hESCs will be necessary to evaluate whether microfibrous scaffolds coated with PDL can support pluripotent hESCs for long periods of time (i.e., more than 10–20 passages). It was suggested that hESC adhesion within microfibrous scaffolds causes enhanced confinement within these scaffolds, which leads to an increase in cell-cell contacts and, subsequently, colony formation. Additionally, the microfibrous scaffolds induce hESCs to deposit large amounts of ECMs, especially those containing laminin [96]. The hESCs appear to have the ability to populate fibrous microenvironments with endogenous ECMs, and ECM deposition may be differentially regulated based on the geometry of the cell culture substrates. Within 3D fibrous substrates [96], the enhanced local surface area relative to that of 2D dishes and 3D macroporous scaffolds [96] allows for the aggregation of hESCs during seeding. With

this aggregation, hESCs can reestablish cell-cell contacts, and endogenously produced ECM components can aggregate, encouraging the generation of undifferentiated hESC colonies [96]. Although the ease of hPSC harvesting from 3D cell culture substrates must be improved, it appears that synthetic substrates with proper 3D microgeometries that contain adhesion molecules, such as PDL, ECMs, or oligopeptides, can support the formation of self-renewing, pluripotent hESC colonies that maintain the ability to differentiate into the three germ layer lineages.

6.5 Three-Dimensional (3D) Culture of hPSCs on Biomaterials

Conventional 2D culture systems, as discussed in the previous sections, are easy to use for hPSC culture in standard operations and are valuable for developing and analyzing the mechanism of the pluripotency maintenance of hPSCs cultured on the biomaterials. Furthermore, the morphology of hPSC colonies (i.e., pluripotency of hPSCs) can be observed by microscopic observation *in situ*. The detachment of hPSCs from biomaterials and the selection of undifferentiated hPSCs from an hPSC colony can be easily performed under microscopic observation. However, the most robust and efficient way to produce hPSCs is the cultivation of hPSCs in a 3D culture system because large numbers of hPSCs must be provided in clinical applications. A minimum of 1×10^9 cells are required per implantation for one patient in current trials using stem cells, such as the trials for treating myocardial infarction. This number corresponds to more than 130 plates of 75 cm^2 T-flasks, supposing a confluent culture of hPSCs with 1×10^5 cells/cm^2.

Several 3D culture strategies for hPSCs have been established: (a) cell culture in macroporous scaffolds, (b) cell culture on nanofibers or microfibers, (c) self-aggregated spheroid (cell aggregate) culture, (d) cell culture on microcarriers, and (e) microencapsulated cell culture in suspended hydrogels [97,98].

Because of the recent development of the ROCK-inhibitor Y27632, as well as the fusion of interleukin-6 receptor to interleukin-6 (IL6RIL6 chimera), hPSCs can be cultured in a single state or on cell aggregates [99–102]. There are several reports dealing with hESC aggregate cultures in suspension [98–102], although this culture method is beyond the scope of this review. Furthermore, the cell aggregate culture of hPSCs in suspension was reported to be less efficient and less reproducible, and this method relied on expensive reagents, ROCK-inhibitors, and a high concentration of bFGF [103]. Therefore, the microcarrier suspension culture of hPSCs or the culture of microencapsulated hPSCs in hydrogels appear to be more suitable than a cell aggregate culture in suspension for 3D culture in a homogenous and scalable stirred 3D culture system [104]. These culture methods are introduced in the following sections.

6.5.1 3D Culture of hPSCs on Microcarriers

Frequently, hPSCs have been cultured on microcarriers in suspension because a high surface-to-volume ratio can be achieved for a microcarrier cell culture system. Microcarriers have been used for the scalable culture of anchorage-dependent cells,

Table 6.6 Research involving expansion of hPSCs cultured on microcarriers. Reproduced with permission from [2]. Copyright 2014 Elsevier Inc.

Cell Line	Microcarrier Name	Materials of Microcarrier	Shape	Coating Materials on Microcarriers	Medium	Fold Expansion (Doubling Time[a])	Longest Culture Time	Ref. (Year)
hESCs (H9)	Cytodex 3	Crosslinked dextran with denatured collagen on surface	Spherical, microporous	None	MEF-CM	6 fold for 4 days	14 days	107 (2009)
hESCs (H1, H9)	Cytodex 3			MEF or Matrigel	MEF-CM	3-fold in 60 h (DT=35h)	11 passages	105 (2009)
hESCs (SCED)	Cytodex 3			Matrigel	MEF-CM with Rock inhibitor	15-fold for 11 days	14 days	110 (2010)
hESCs (HES-2, HES-3)	Cytodex 1	Diethylaminoethyl (cationic) dextran	Spherical	laminin or Matrigel	MEF-CM	20-fold for 7 days	10 passages	65 (2011)
hESCs (ESI-017, HUES9)	Hillex II	Crosslinked PSt modified with cationic trimethyl ammonium	Spherical	None	MEF-CM	3-fold for 5 days	6 passages	106 (2008)
hESCs (H9)	Hillex II		Spherical	None	MEF-CM	DT=30.3h	264h	103 (2013)
hESCs (HES-2, HES-3)	DE53	Diethylaminoethyl (cationic) cellulose	Microgranular cylindrical	Matrigel	mTeSR1, StemPro	10-fold for 7 days (DT=21 h)	7 days	111 (2009)
hESCs (HES-3). hiPS (IMR90)	DE53		Microgranular cylindrical	Matrigel	mTeSR1	10-21 fold for 7 days (DT=31-36 h)	10 passages	112 (2013)
hESCs (HES-2, HES-3)	DE53		Microgranular cylindrical	laminin or Matrigel	MEF-CM	20-fold for 7 days	10 passages	65 (2011)

(*Continued*)

hESCs (HES-3, H7)	Not specified	Crosslinked PSt microcarrier	spherical	LN or VN	StemPro	8.5-fold for 7days (DT=25h)	20 passages	113 (2012)
hESCs (SHEF-3)	Cultispher-S	Crosslinked porcine gelatin	Spherical, macroporous	None	KO-DMEM with KSR, bFGF, periodi-cally used Y-27632	5-10 fold for 7 days	2 passages	108 (2010)
hESCs (H1,H9)	Hyclone	Collagen-coated microcarriers	Spherical	Matrigel	MEF-CM	13-fold for 8 days (DT=36.7h)	12 days	109 (2009)

[a]DT, doubling time

including human retinal pigment epithelial cells and human hepatocytes, and for the co-culture of neurons and astrocytes [105]. Microcarriers are typically spherical particles composed of a variety of materials, including cellulose, polystyrene, glass, and polyester, with a typical diameter of 100–250 μm [105]. Recent attempts to culture hPSCs on microcarriers are summarized in Table 6.6 [65,103,106–113]. Microcarrier systems provide the flexibility of culturing cells either within a macroporous scaffold or on the surfaces of the microcarriers.

Phillips *et al.* investigated the attachment and expansion of hESCs (ESI-017) on microcarriers made of several types of polymers with various morphologies in MEF-CM (Figure 6.13 and Table 6.6) [106]. The microcarriers were used without MEF attachment or coating by an ECM or Matrigel. The hESCs could attach to the Hillex II microcarriers made of the crosslinking polystyrene modified with triethyl ammonium that had cationic charged surfaces and to the Cultisphere that had cationic charged surfaces. In contrast, hESCs could not adhere to the Cytodex 3 microcarriers made of crosslinked dextran or to the Solohill plastic+ or Solohill pronectin microcarriers. Solohill plastic+ is made of crosslinked polystyrene microcarriers with a cationic charge, and Solohill pronectin contains pronectin-coated polystyrene microcarriers [106]. Pronectin is an oligopeptide containing the RGDS sequence and is designed to mimic fibronectin. Relatively few hESCs adhered to the Cytodex 1 microcarriers, which are made of crosslinked dextran with a diethylaminoethyl group and have cationic charged surfaces [106]. In general, the positively charged surfaces of microcarriers such as Hillex II promote hESC adhesion on the surfaces by electrostatic interactions because the cells have

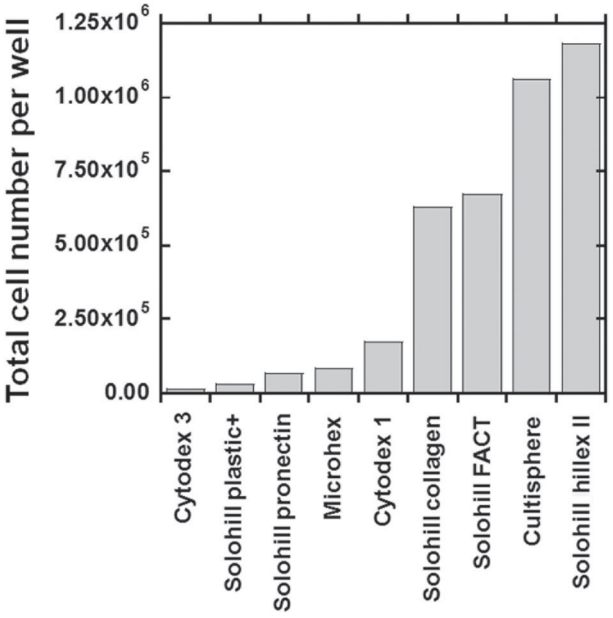


Figure 6.13 Attachment and growth of ESI-017 hESCs on microcarriers. The number of attached ESI-017 cells cultured on nine different microcarriers in MEF-CM after a 72 h culture. The initial seeding number was 1.0×10^6 cells. This figure was modified with permission from [106]; Copyright 2008 Elsevier Inc.

negatively charged surfaces in the resting state. However, other factors, such as the size, shape, and materials of the microcarriers, also appear to affect the hESC adhesion to the microcarriers because, in that study, some microcarriers with positively charged surfaces did not show good hESC adhesion to the microcarriers (e.g., Cytodex 1) [106]. A collagen coating on the microcarriers (Solohill collagen) did not promote better hESC adhesion compared with the adhesion using the cationic charged microspheres (Hillex II). The microcarriers used in this work were not coated with Matrigel, laminin, or vitronectin; therefore, the hESCs could not expand for more than three or six passages, and the expansion period depended on which cell detachment enzyme, collagenase or trypsin, was used.

Several other researchers reported hESCs to be cultured on microcarriers without a Matrigel or ECM coating [103,107,108]. However, the hESC culture period of those studies was reported to be a relatively short period (no more than two to six passages). Therefore, most researchers [65,105,113] currently use a Matrigel or ECM coating on the microcarriers to expand hPSCs and to maintain their pluripotency.

Nie *et al.* investigated hESC (H1 and H9) adhesion and cell viability on several microcarriers in MEF-CM after five days of culture; the microcarriers were made of crosslinked dextran, gelatin, and polystyrene and had different surface segments and charges [105]. The microcarriers were precoated with laminin and fibronectin to promote hESC adhesion on the microcarrier surfaces. A visual assessment of hESC attachment and cell viability is shown in Table 6.7 [105]. hESC survival was not observed for several types of crosslinked polystyrene microcarriers (e.g., H11-921, F102, C102, G102, P102, PP102, and PF102) coated with laminin and fibronectin. Crosslinked dextran microcarriers (Cytodex 1 and 3) coated with laminin and fibronectin promoted hESC adhesion. In particular, the hESCs that were cultured on crosslinked dextran microcarriers with denatured collagen (Cytodex 3) and coated with laminin and fibronectin showed fair adhesion on the microcarriers with high cell viability in MEF-CM [105].

Chen *et al.* also investigated the effects of ten types of microcarriers on the attachment efficiency, cell growth, and pluripotency of hESCs (HES-2 and HES-3) in MEF-CM; the microcarriers were coated with and without ECMs or Matrigel (Table 6.6) [65]. The microcarriers in their study were (a) cylindrically shaped cellulose microcarriers with positively charged (DE52, DE53, and QA52) and negatively charged surfaces (CM52), (b) spherically shaped crosslinked dextran (Cytodex 1, Cytodex 3), (c) crosslinked gelatin microcarriers (Cultispher G), (d) spherically shaped cellulose microspheres (Cytopore 2), and (e) spherical hydroxylated methacrylate microcarriers (Tosho 65 PR and Tosoh 10 PR) [65]. The hESCs showed less cell attachment (38%) on smaller (10 μm) microcarriers (Tosho 10 PR). The macroporous gelatin microcarriers (Cultispher G) showed low hESC attachment efficiency (23%) [65]. This finding is most likely due to the shortage of nutrients for the hESCs in the macropores due to the limited diffusion of nutrients into the microcarriers. The hESCs on negatively charged (CM52) microcarriers were not able to support cell growth and pluripotency. Only the hESCs on large spherical microcarriers (Cytodex 1, Cytodex 3, Cultispher G, and Cytopore 2) could grow with 67–85% expressing the pluripotent surface marker Tra-1-60 at passage 3 when the microcarriers were not coated with ECM or Matrigel in MEF-CM [65]. Therefore, the hESCs were

Table 6.7 Preliminary screening of hESC culture on some microcarrier materials coated with laminin and vitronectin in MEF-CM after 5 days. Reproduced with permission from [2]. Copyright 2014 Elsevier Inc.

Microcarrer	Material	Cell Attachment	Cell Viability
Cytodex 1	Cross-linked dextran with N, N-diethylaminoethyl groups	Fair	Poor
Cytodex 3	Crosslinked dextran immobilized with denatured collagen on surface	Fair	Fair
Cytopore 1	Macroporous cross-linked dextran with N,N-diethyl aminoethyl groups, charge density of 1.1 meq/g	Poor	Poor
Cytopore 2	Macroporous cross-linked dextran with N,N-diethyl aminoethyl groups, charge density of 1.8 meq/g	Poor	Poor
CultiSphere-S	Crosslinked pharmaceutical grade porcine gelatin	Fair	Poor
Solo Hill H11-921	Crosslinked polystyrene modified with cationic trimethyl-ammonium	Poor	None
Solo Hill F102	Crosslinked polystyrene modified with cationic gelatin	Poor	None
Solo Hill C102	Crosslinked polystyrene modified with gelatin	Poor	None
Solo Hill G102	Crosslinked polystyrene modified with high silica glass	Poor	None
Solo Hill P102	Crosslinked polystyrene	Poor	None
Solo Hill PP102	Cationic crosslinked polystyrene	Poor	None
Solo Hill PF102	Crosslinked polystyrene modified with recombinant fibronectin	Poor	None

able to be cultured on microcarriers coated with Matrigel; the hESCs showed long-term growth and maintained their pluripotency in that study. Most microcarriers showed decreased cell attachment efficiency for coatings with Matrigel. For example, the cell attachment efficiency on positively charged DE53 and QA52 microcarriers decreased by 11% and 18%, respectively [65]. The reduced cell attachment efficiency was attributed to the Matrigel coating, which led to the masking of the positively charged surfaces on the microcarriers. However, the Matrigel coating on the microcarriers significantly improved the cell yield and pluripotency in long-term cultures

(3–11 passages); these cultures showed more than 90% viability, except for the hESCs cultured on negatively charged microcarriers (CM52) and on microcarriers composed of gelatin (Cultispher G) [65]. Finally, positively charged cylindrical cellulose microcarriers (DE53) and positively charged spherical microcarriers (Cytodex 1) were selected for the subsequent investigation due to the high hPSC expression of pluripotent markers while using the microcarriers in MEF-CM. In contrast to the Matrigel coatings, the laminin-coated microcarriers (DE53 and Cytodex 1) were able to support long-term propagation with high pluripotency of hESCs in MEF-CM (Figure 6.14 [65]). The cell yield of hESCs cultured on fibronectin-coated microcarriers was found to be slightly less than that on laminin, and the cell yield of hESCs on vitronectin or hyaluronic acid was less than that on laminin or fibronectin [65]. This finding suggests that a Matrigel or laminin coating is essential for the stable long-term propagation of hESCs (ten passages) on specific microcarriers (e.g., DE53 and Cytodex 1) in MEF-CM, whereas conventional microcarriers without a coating of Matrigel or ECMs cannot support hESC expansion while maintaining the hESC pluripotency for more than three passages.

In most cases, hPSC cultures on microcarriers require a Matrigel coating and/or MEF-CM. These conditions are not xeno-free culture conditions and make it difficult to use hPSCs in clinical applications.

Heng *et al.* cultured hESCs (HES-3 and H7) on crosslinked polystyrene microcarriers (7602B, Thermo Fisher Scientific) coated with vitronectin or laminin in chemically defined StemPro medium (Table 6.6) [113]. The adsorption of vitronectin and laminin onto the microcarriers resulted in coating surface densities of 450 and 650

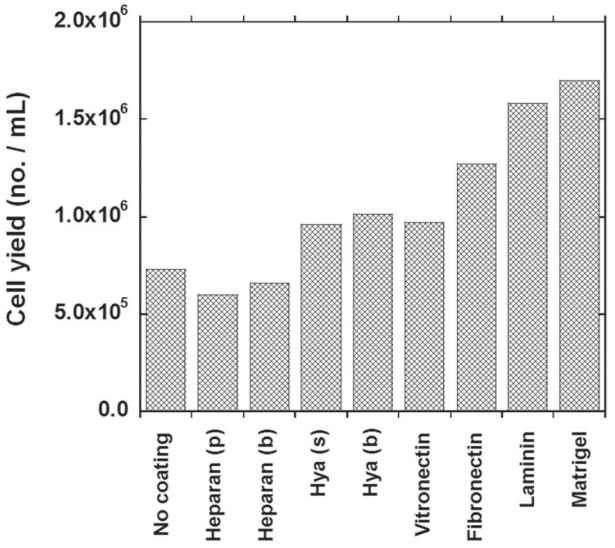


Figure 6.14 hESC (HES-3) propagation on cellulose microcarriers (DE53) coated with different ECM components in MEF-CM after two passages where heparan sulfate (p) indicates heparan sulfate derived from pig, heparan (b) is from bovine, Hya (s) indicates hyaluronic acid derived from *Streptococcus*, and Hya (b) is from bovine. In total, hESCs at 1.6×10^5 cells/ml were seeded on a 4 mg/ml microcarrier. This figure was modified with permission from [65]; Copyright 2012 Elsevier Inc.

ng/cm^2, respectively, which were used to support hESC propagation [113]. The long-term expansion of hESCs was found to be possible on the microcarriers coated with vitronectin or laminin with a high expression (>90%) of pluripotent markers (Oct-4 and TRA-1-60) for 20 passages while maintaining normal karyotypes. The average fold increase in cell numbers on the microcarriers coated with both vitronectin and laminin per serial passage was 8.5 [113]. Embryoid body differentiation assays and teratoma formation confirmed that the hESCs retain their ability to differentiate into lineages of all three germ layers. This work is the first report in which hESCs were expanded on microcarriers for long passages (20 passages) under feeder layer-free and xeno-free culture conditions using chemically defined medium.

Although Heng's work [113] achieved feeder layer-free and xeno-free cultures of hESCs in chemically defined medium, the researchers only verified a few ES cell lines (HES-3 and H7). It is not clear whether the microcarriers and cell culture protocol used by Heng *et al.* could support all hESC and hiPSC lines. It is preferable to prepare or synthesize microcarriers composed of other types of biomaterials and to develop and design optimal microcarriers for hPSC culture in feeder layer-free and xeno-free culture conditions by referencing Heng's work [113].

6.5.2 3D Culture of hPSCs Entrapped in Hydrogels (Microcapsules)

The microencapsulation of hPSCs is an attractive technology that protects against hydrodynamic shear and prevents excessive aggregate agglomeration while allowing for the efficient diffusion of nutrients, gases, and growth factors through the microcapsule walls (Figure 6.15) [114]. Several hydrophilic biomaterials (hydrogels) can be selected to entrap hPSCs in the hydrogels; these materials include alginate, hyaluronic acid, agarose, dextran, and polyethylene glycol derivatives [97,115,116].

Alginate is the most common encapsulation material. Alginates are unbranched binary copolymers of 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid

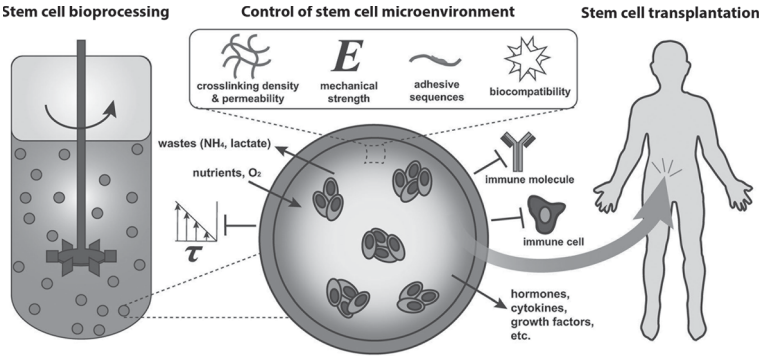


Figure 6.15 Microencapsulation of hPSCs. Microencapsulation of hPSCs permits the mass transport of nutrients and secretory products while restricting the passage of immune molecules and cells and shielding them from physical forces. The modification of various capsule parameters can modulate stem cell response(s) while simultaneously enabling expansion in scalable suspension bioreactors for bioprocessing and transplantation *in vivo.* Reproduced with permission from [114]; Copyright 2012 Wiley Periodicals, Inc.

Table 6.8 Research involving expansion of hPSCs microencapsulated in hydrogels (biomaterials). Reproduced with permission from [2]. Copyright 2014 Elsevier Inc.

Cell Line	Materials of Microencapsulation	Shape	Medium	Fold Expansion	Longest Culture Time	Ref. (Year)
hESCs (H1)	Alginate-gelatin hydrogels	Spherical (2.3 mm diameter)	DMEM with KSR and bFGF	Not specified	260 days	83 (2008)
hESCs (SCED)	Alginate hydrogel	Spherical	MEF-CM	19.2 for 19days	19days	97 (2011)
hESC (H1, H9)	PEG-based hydrogel	Spherical	DMEM/F12 with KSR, bFGF	1.3-1.7 fold for 10 days	10 days	118 (2013)
hESCs (H1, H9, H13)	Crosslinked methacrylated hyaluronic acid	discs with diameters of 3 mm and thicknesses of 2 mm	MEF-CM	4-fold for 4 days	20 days	104 (2007)

(G) of widely varying composition and sequential structures. Alginate gelation occurs when multivalent cations, such as Ca_2^+, ionically interact with blocks of guluronic residues between two different chains; this interaction results in a three-dimensional network, which leads to the generation of hydrogels [117]. Therefore, hPSCs encapsulated in an alginate hydrogel can be prepared by dropping an aqueous alginate solution containing hPSCs into a $CaCl_2$ solution. Alginate possesses several favorable properties, such as biocompatibility, biosafety, and permeability, that are required in biomaterials. However, alginates possess bioinert characteristics and do not specifically interact with hPSCs. Consequently, some researchers have added ECMs or oligopeptides containing cell binding domains to alginate hydrogels using a blending or grafting method. Recent attempts to culture hPSCs that were microencapsulated in hydrogels are summarized in Table 6.8.

Siti-Ismail *et al.* encapsulated hESCs (H1) in alginate hydrogels containing gelatin through the dropwise addition of an aqueous alginate solution containing hESCs and gelatin into a $CaCl_2$ solution using a 25-gauge needle [83]. The hESCs in the alginate hydrogels could be cultured in DMEM supplemented with 20% knockout serum replacement (KSR) for a period of up to 260 days. Cell aggregates were formed within the alginate hydrogels that retained pluripotency and could differentiate into the three germ layer lineages when they were subsequently cultured in a differentiation medium [83]. These data suggest that hESCs can be maintained in an undifferentiated state in a hydrogel without passaging for a long period of time. However, the expansion (fold increase) and viability rate of the hESC proliferation in the hydrogels were not examined in this study. It was also unclear whether the growth of hESCs in the alginate hydrogels was decreased or arrested or whether the cells proliferated at a normal rate. However, hydrogels that contain adhesion sites, such as ECMs, that can be blended into hydrogels might represent promising matrices for hPSC culture.

Serra *et al.* evaluated various 3D culture strategies for the encapsulation of hESCs as (a) single cells, (b) cell aggregates, and (c) cells adhered on microcarriers in alginate hydrogels (Figure 6.16) [97]. The microencapsulated hESCs were cultured in stirred tank bioreactors. Microencapsulation improved the culture of hESC aggregates (30–100 μm) by protecting the cells from hydrodynamic shear stress, controlling the aggregate size, and maintaining the cell pluripotency for two weeks; in contrast, the cell viability decreased gradually from approximately 95% to 5% after seven days of cultivating single hESC cells entrapped in alginate microcapsules [97]. This finding suggests that the microencapsulation of single cells is not a suitable protocol for hESC expansion while maintaining pluripotency. A significant improvement in cell viability and metabolic activity in hESC aggregates in alginate microcapsules was observed compared with those in the non-encapsulated hESC culture.

For the (c) protocol, hESCs were immobilized on Matrigel-coated microcarriers (Cytodex 3) and encapsulated in spherically shaped alginate hydrogels. The microencapsulation of hESC-adhering microcarriers in the alginate hydrogels significantly enhanced the cell viability and cell expansion when compared with those in non-encapsulated 2D hESC culture [97]. The combination of microencapsulation and microcarrier technology resulted in the high production and storage of pluripotent hESCs. This method leads to high cell expansion, such as the 19-fold increase in cell concentration, when hESCs have enough surface area to grow (Table 6.8) [97].

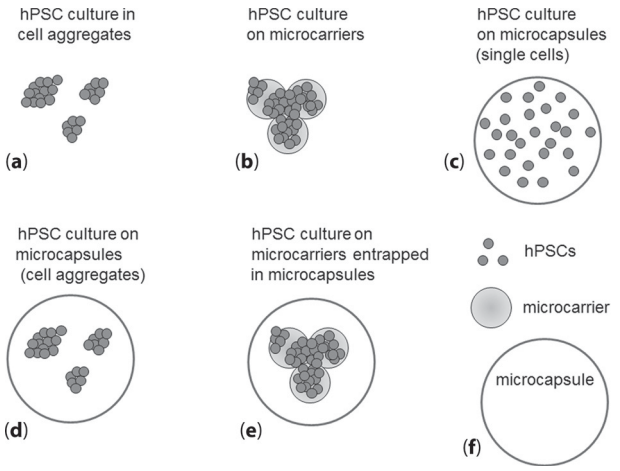


Figure 6.16 Conceptual model of hPSC culture in 3D. hPSCs can be cultured (a) in cell aggregates, (b) on microcarriers, (c) on microencapsulated single cells, (d) on microencapsulated cell aggregates, and (e) on microcarriers entrapped in microcapsules. Figure modified with permission from [97]; Adapted 2012. Reproduced with permission – a Creative Commons Attribution License.

This study suggests that the combination of cell microencapsulation and microcarrier technology results in an optimal process for the scalable production of high-quality pluripotent hPSCs because microencapsulation ensures a shear stress-free microenvironment and avoids the excessive clustering of microcarriers and aggregates in hPSC culture.

The alginate encapsulation of hPSCs typically results in weak mechanical properties and chemical instability in addition to poor resistance to osmotic swelling, which often results in cell escape, although Siti-Ismail *et al.* reported a 260-day culture of hPSCs encapsulated in alginate hydrogels [83]. Therefore, the exterior of alginate microcapsules is often coated to increase the mechanical stability by polyelectrolyte complexation, where two oppositely charged polymers are complexed together [114]. The coating materials are polycations, such as poly-L-lysine (PLL), because of the polyanionic characteristics of alginate. PLL binds to both the M and G blocks of alginate through ionic interactions, and PLL typically binds more tightly to alginates with higher M content [114]. To improve biocompatibility, the positive PLL charges on the exterior of the microcapsule are neutralized through deposition of additional alginate, forming alginate-PLL-alginate microcapsules. Chitosan, polyethylene glycol (PEG), and other polycations might be used as alternatives to PLL [114].

Jang *et al.* developed a 3D synthetic polyethylene glycol (PEG)-based hydrogel microcapsule using PEG3400 for hESC (H1, H9, and Novo) culture in a feeder layer-free condition (Table 6.8) [118]. The PEG-based hydrogels were prepared by the conjugation of vinylsulfone-functionalized 3-arm PEG, 4-arm PEG, and 8-arm PEG to dicystein-containing peptides with an intervening matrix metalloproteinase (MMP)-specific cleavage site (Ac-GCRD-GPQGIWGQ-DRCG-NH2) through a Michael-type addition reaction (Figure 6.17) [118,119]. A high expression of MMP-2 in H9 hESCs

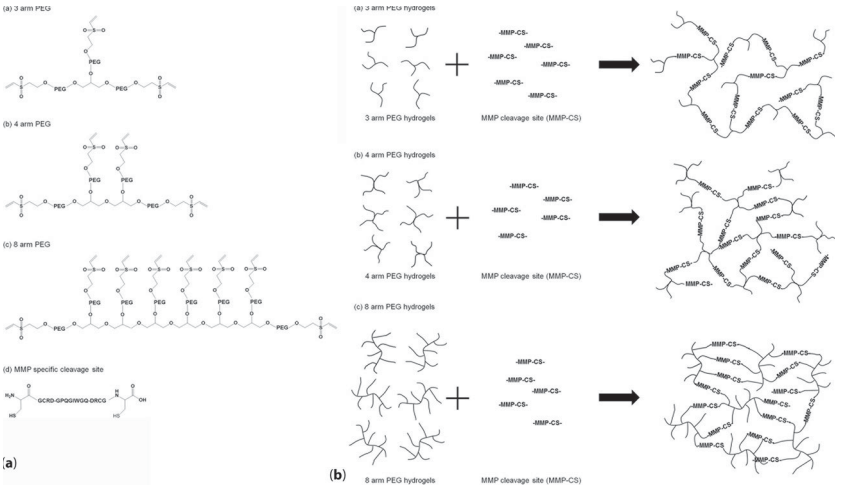


Figure 6.17 Chemical structures of PEG-based hydrogels. A. Chemical structures of a 3-arm PEG (a), 4-arm PEG (b), 8-arm PEG (c), and an oligopeptide with an MMP-specific cleavage site (d). B. Chemical reaction of 3-arm PEG hydrogels (a), 4-arm PEG hydrogels (b), and 8-arm PEG hydrogels (c). Reproduced with permission from [2]; Copyright 2014 Elsevier Inc.

was observed compared with that using other MMP isomers. Therefore, the researchers selected the MMP-2-sensitive PEG-based hydrogel for hESC growth in DMEM/F12 medium with KSR and bFGF. The physical properties of the stem cell microenvironment are important factors for determining cell fate and for maintaining the pluripotency and differentiation of stem cells [35], as discussed in Section 6.3. Significant effects of PEG concentration (5, 7.5, 10, 12.5, or 15%) and vinyl sulfone-functionalized PEG multiarm number (3, 4, or 8) on hESC morphology were observed in this study [118]. The PEG arm numbers and PEG concentration affected the stiffness of the PEG-based hydrogels. The hESCs underwent apoptosis in the 3-arm PEG-based hydrogels, which were softer hydrogels because of the shortage of hydrogel crosslinking compared with that in the 4-arm and 8-arm PEG-based hydrogels [118]. The relatively stiffer hydrogels from the 4-arm and 8-arm PEG-based hydrogels appear to be preferable for hESC cultures that maintain cell viability and pluripotency.

Cell growth was found to be maximal with the 8-arm architecture and 10% PEG concentration, which generated the highest expression of stemness-related genes (Oct3/4, Nanog, Klf4, Tert, Sox2) on hESCs [118]. The 8-arm PEG-based hydrogels consisting of 10% PEG were considered to be the optimal condition for the culture in the feeder-free 3D conditions. The alkaline phosphatase activity in cultured H9 cells in the 8-arm PEG-based hydrogels was similar to that in the 2D culture of conventional MEF feeder cells [118]. However, an increased expression of pluripotent genes (Klf-4, Cdh1, Tert, Sox2, and Utf1) and higher expression of pluripotent surface markers (SSEA-4, Oct3/4, Nanog, Tra-1-60, and Tra-1-81) were detected in the 3D-cultured hESCs compared with those in the hESCs cultured on MEF feeder cells (2D culture) [118]. These results indicate that the chemically defined, acellular niches developed using PEG-based hydrogels have the ability to support hESC self-renewal.

6.6 hPSC Culture on PDL-Coated Dishes with the Addition of Specific Small Molecules

Rho signaling is required for the maintenance of cell-cell contacts in hESCs [120]. The use of a ROCK-specific inhibitor revealed that cell-cell adhesion was reversibly controllable and indispensable for ESC self-renewal. Harb *et al.* [120] and Meng *et al.* [31] reported that hESCs could be cultured on PDL-coated dishes in defined mTeSR1 medium supplemented with the ROCK inhibitor Y27632. These hESCs maintained their pluripotency, their ability to self-renew, and their ability to differentiate into the three germ layer lineages. In conventional cultures, Y27632-containing medium is usually replaced with fresh medium without Y27632 24 h after seeding or thawing dissociated cells because Y27632 can promote hPSC attachment and viability but not proliferation [31]. However, Y27632 was always present in the culture medium in these experiments [31] because the hESCs tended to detach from the culture surfaces if refeeding occurred without Y27632 in the culture medium.

Long-term culturing (e.g., > 10–20 passages) of hESCs on PDL-coated dishes in defined medium supplemented with Y27632 should be performed to evaluate whether hPSCs can proliferate and maintain their pluripotency and ability to differentiate into the three germ layer lineages under these conditions.

6.7 Conclusion and Future Perspective

Human pluripotent stem cells (hPSCs) are commonly cultivated on MEF or human feeder cells in a culture medium containing KSR or in mTeSR1, StemPro, or another commercial culture medium used for feeder-dependent cultures. After a few passages, the hPSCs can be shifted into a feeder-free culture system consisting of Matrigel- or Geltrex-coated dishes with mTeSR1, StemPro, or another commercial culture medium. Neither type of culture system is suitable for clinical trials as pharmaceuticals because serum, MEF, or Matrigel, which is derived from xeno-origin is used.

Currently, some feeder-free and xeno-free culture systems are commercially produced: hPSCs are able to proliferate on (1) vitronectin-coated dishes in TeSR2 medium; (2) Synthemax (an oligopeptide derived from vitronectin) dishes in TeSR2, StemPro medium, or another hPSC culture medium; (3) CELLstart (mixture of albumin and fibronectin)-coated dishes in TeSR2, StemPro, or another hPSC culture medium; (4) StemAdhere-coated dishes in TeSR2; or (5) PDL-coated dishes in TeSR2 supplemented with Y27632. However, not all hPSCs can proliferate and maintain their pluripotency in each of the above serum-free culture systems. Further development of coating materials or cell culture biomaterials, including nanofibers, microfibers, microcarriers, and hydrogels, is demanded for the long-term culturing of pluripotent hPSCs as well as for the development of an optimal hPSC culture medium for their pharmaceutical usages.

The clinical applications of hPSCs will require hPSC production on a large scale. Currently, hPSC culture media and culture dishes are extremely costly; thus, the large scale production of hPSCs is extremely difficult. Furthermore, the surface design of

microcarriers or microfibers for use in the mass production of pluripotent hPSCs in bioreactors should be considered. The current microcarrier materials used for hPSC culture are limited to crosslinked polystyrenes, dextran, and gelatin beads because the typical microcarriers used for hPSC culture are selected from commercially available beads (Tables 6.6 and 6.7). However, in 2D culture system, some sophisticated biomaterials have been reported for the long-term culture of hPSCs; these biomaterials are oligopeptide-immobilized dishes and completely synthetic dishes under feeder layer-free and xeno-free culture conditions. In this case, ECM-coated microcarriers or microfibers will not be adequate for hPSC production because of the high cost of ECMs. Synthetic microcarriers, nanofibers, microfibers, or hydrogels that support hPSC proliferation while maintaining their pluripotency might be promising in the future. A topic of interest will be the development of microcarriers prepared from a surface (or polymeric) design that holds hPSC expansion and keeps hPSC pluripotency for a long-term 2D culture. Microcarriers that are coated with specific polymers (PMEDSAH, PMVE-alt-MA, APMAAm, and AEtMA-Cl/DEAEA) or grafted with specific oligopeptides (VN-PAS, Oligo-HBP1) are expected to maintain several types of hPSCs while keeping pluripotency in a chemically defined medium for the long term (more than 20 passages).

Currently, no systematic research has been performed to determine why certain synthetic polymers with a specific chemical scheme support pluripotency of certain hPSC cell lines. Some specific polymers that have been reported to maintain hPSC pluripotency are still necessary to be reevaluated using different cell lines and culture media, and the results should be evaluated by different researchers. It is still unclear whether completely synthetic surfaces can support hPSC pluripotency in xeno-free culture conditions without an ECM, oligopeptide, or polysaccharide coating; indeed, most synthetic surfaces appear to require the secretion of ECMs from hPSCs or the adsorption of proteins from a conditioned medium of feeder cells (or another culture medium). Biological assays examining synthetic surfaces and surface coatings that support hPSCs will help to uncover the most valuable and efficient synthetic surface designs or coating biomaterials for hPSC culture.

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7

New Perspectives on Herbal Nanomedicine

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Abstract

Over the past few years, there has been an in-depth analysis of drug delivery using novel carrier systems. Novel methods like nanoparticles, nanocrystals, nanocapsules, microemulsions and phytosomes have improved the solubility, bioavailability, duration of action and, alternatively, therapeutic effectiveness of drugs. Ayurveda and Siddha are the traditional systems of medicine which have been followed by Indian Ayurvedic practitioners for the last thousand years, whose potential has just being realized within the last few years. However, the drug delivery system used for administering herbal medicine to the patient is ancient and noncurrent, resulting in reduced efficaciousness of the drug. If the novel drug delivery technology is applied in herbal medication, it should facilitate in increasing the efficaciousness and reducing the facet effects of varied herbal compounds and herbs. This is the basic plan behind incorporating novel methodology of drug delivery in herbal medicines. However, contemporary phytopharmaceutical analysis will solve the scientific desires (such as determination of pharmacological effect, mechanism of action, site of action, correct dose required, etc.) of herbal medicines to be incorporated in novel drug delivery systems, such as nanoparticles, matrix systems, solid dispersions, liposomes, solid lipide nanoparticles and so on. This review summarizes numerous drug delivery technologies which may be used for herbal actives along with some examples.

Keywords: Herbal drug, nanogreen, nanoparticle liposomes, curcumin, phytopharmaceutical

7.1 Introduction

Over the last thousand years, herbals have been used by humans in the form of constituents, herbal extracts and finished herbal products; other uses may be in functional foods and meditative products, as well as in opiates, perfumes, decorative agents, dyes, poisonous arrows and for salvation. With the development of chemical structures, the pharmacology and mechanisms of action of plant active constituents have resulted in the expansion of life-saving medicines for human diseases. For human civilization, many herbal medicines have saved the lives of millions of people, some examples of

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which are *Digitalis purpurea,* which is used for cardiovascular disease [1]; *Cinchona officinalis,* used as the first antimalarial drug; Reserpine, the first antihypertensive drug, was isolated in 1952 from the dried root of *Rauwolfia serpentina;* vincristine from *Catharanthus roseus* [2] and paclitaxel from the *Taxus brevifoliais* are used as antineoplastic drugs [3]; *Momordica charantia* is used as a hypoglycemic drug [4]; and the first antibiotic penicillin was from *Penicillium notatum* [5]. In the last hundred years, due to herbal drug innovation and expansion, various helpful drugs have been developed, creating a great many successes in curing human diseases. Still, there is a pressing need for a lot of efficacious medicine [6]. Herbal medicines are the combination of therapeutic experiences for the generation of autochthon systems of medication for many years. The majority of biologically active constituents in plants are water soluble in nature, like flavonoids, glycosides, tannins, etc. These constituents are weakly absorbed either owing to their giant molecular mass that cannot be absorbed by inert transmission or owing to their reduced macromolecule solubility, a factor that severely restricts their capability to pass through the lipid-rich natural membranes, following reduced bioavailability [7].

The usage of standardized extract within the production of herbal medication has become more and more common and fashionable. However, throughout the extraction method the drug molecules or active elements within the herbal medication are exposed to oxidization, a reaction to different environmental degradations [8]. Nanocoatings of those drug molecules protect the active chemicals from degradation and thus enhance the time period of the herbal product. So the integration of herbal drug into novel drug delivery systems has certain adscititious benefits, like overcoming poor absorption and high dosage, which are the most common problems for all herbal drugs and are attractive to all national and international pharmaceutical companies [9].

The word "nanotechnology" was initially used in 1974 by the late Norio Taniguchi and was inspired by an idea that a nuclear physicist had in 1959 [10,11]. Nanotechnology is conducted at dimensions at a nanosclae, which is 10^{-9} of a meter; it is the power to figure at the molecular level to form giant structures with essentially new molecular organization. The interaction of nanotechnology's building blocks and inter-atomic forces has the power to manage and manipulate the physical, biological and chemical properties of a system. The nanocarriers are manufactured from safe materials, as well as artificial perishable polymers, lipids and polysaccharides. These novel carriers should preferably meet some conditions. Firstly, the amount of drug released should be directly proportional to the need and duration of the cure; secondly, the herbal drug should reach the appropriate target [12]. The novel herbal drug release method targets the site of action and the amount of drug release is directly proportional to the need and duration of the cure.

7.1.1 Novel Herbal Drug Formulations

Various novel herbal drug formulations are depicted in Figure 7.1. They are:

1. Vascular delivery methods: Transferosomes, Ethosomes, Liposomes, Phytosomes;

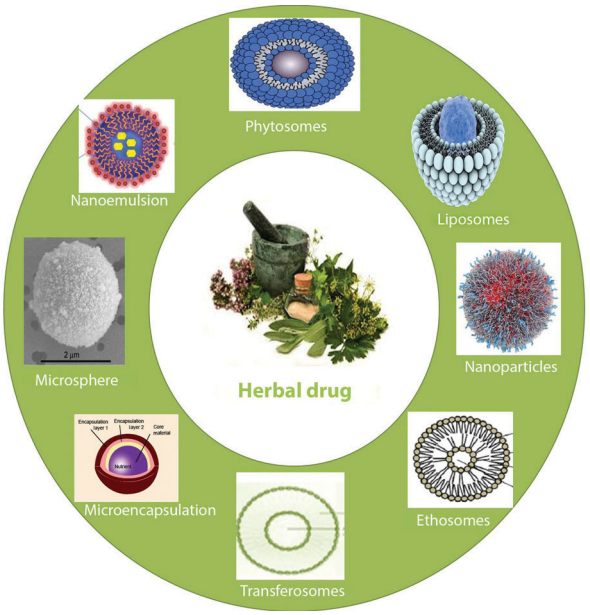

Figure 7.1 Various novel herbal drug formulations.

2. Nano Drug delivery methods: Micropellets, Microspheres, Nanoparticles;
3. Biphasic Drug delivery methods: Micro/Nano emulsions.

7.2 Phytosomes

The word "phyto" stands for plant, while "some" represents cellular. Phytosomes are extremely tiny cells resembling structure. Phytosomes are often a highly developed type of herbal drug delivery system that contains the active constituents of herb surrounded with supermolecule. Plant secondary metabolites like flavonoids, glycosides and tannins are water soluble in nature, and from all of these water-soluble metabolites, flavonoids are a broad category of biological active secondary compounds having significant therapeutic value. The attributes of aqueous herbal extract and outer lipid layer phytosomes provide higher absorption, good bioavailability and long duration of action as compared to simple herbal formulations [14]. Phytosomes produce lipid membrane surrounding the herbal drug which protects against the degradation of herbal drug by biological enzymes, acid and intestinal microorganisms. Phytosomes are able to transfer the extract from deliquescent atmosphere into the lipid atmosphere of the plasma layer and then finally get to the blood via cell. These formulations are prepared by reacting herbal extract or herbal constituents with soy phospholipids in appropriate solvents. Phytosomes have varied physical, chemical and spectroscopic properties having different novel applications [15]. Moreover, phytosomes have been used in several widespread herbal extracts together with ginko, silybum, liquorice and ginseng [16]. The secondary metabolites like flavonoids present in these phytomedicines are easily attached with phophatidylcholine and form complex [17].

Table 7.1 Different commercial phytosomes preparations [21–24].

Herbal Source	Phytoconstituent	Medicinal Uses
Milk Thistle	Sylimarin	Antihepatotoxic
Maidenhair Tree	24% Ginkgoflavonoids	Brain tonic
American ginseng	Ginsenosides	Adaptogenic
Tea Leaves	Catechin	Treatment of Cancer
Olive oil	Phenolic Compounds	Treatment of inflammation
Grape Vine	Procyanidins	Nutraceutical, systemic antioxidant, a
Thornapple	Flavonoids	Nutraceutical, cardio-protective
Purple coneflower	Echinacosides	Immunostimulants
Indian Pennywort	Triterpenes	Nervine tonic, Skin disease

Yanyu and coworkers prepared the phytosome of silymarin and tested its different pharmacological action. During their study the absorption of silymarin inflated significantly, and the therapeutic activity was also increased in a reduced dose [18]. Ravarotto and coworkers (2004) prepared silymarin phytosome and tested it for antihepatotoxic activity; and he showed that silymarin phytosomes have more potent antiheapatotoxic activity than silymarin extract alone. Meriva® is a patented phytosome formulation of curcumin with soy phospholipids. The two compounds form a noncovalent adduct during a 1:2 weight quantitative relation, and two components of crystalline polysaccharide are supplementary to enhance formulation, with overall contents of curcumin of 20% [19]. Phytosome of glycyrrhetinic acid is the complex sort of glycyrrhetinic acid with phosphatidyl choline. The active part of glycyrrhetinic acid is structurally almost like Cortisol, a hormone with anti-inflammatory drug properties, and potentiates its activity by inhibiting its internal organ and inactivation of animate things [20]. Various commercial phytosome preparations are presented in Table 7.1.

7.3 Liposomes

Liposomes are composed of a double layer in which one layer is made up of phospholipid and the internal layer is water-soluble drug (Figure 7.2). The liposome layer may be composed of naturally-derived phospholipids with mixed supermolecule chains and a variation of head group or of untainted artificial lipids with definite acyl chains and head groups. The phospholipids align themselves side-by-side with their oleophilic heads familiarizing themselves with one another. Medication with a wide variety of lipophilicities may be encapsulated within the liposomes, either within the phospholipids bilayer, within the entrapped binary compound volume or at bilayer interface.

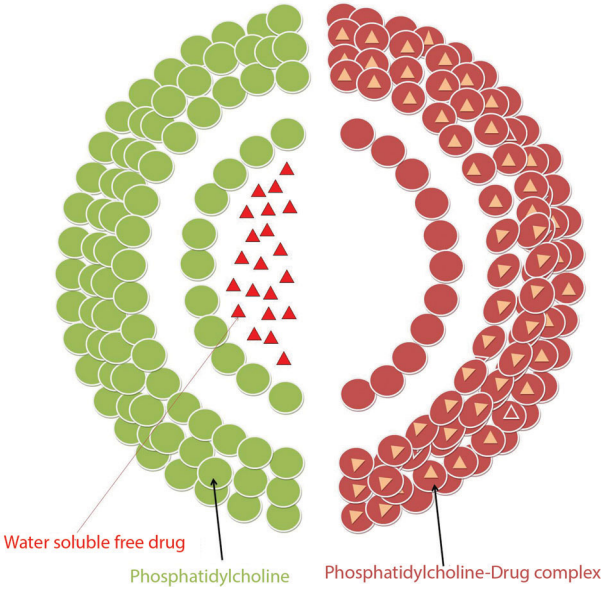

Figure 7.2 The structural arrangements of the liposome (left side) and phytosomes (right side).

Liposomes are sometimes shaped from phospholipids and are the accustomed modification of the pharmacokinetic profile of not exclusively medication, but also herbs, enzymes, etc. [25].

7.3.1 Classification of Liposomes by Work and Mode of Delivery

Liposomes are classified according to their work and mode of delivery as:

i. Classic liposomes;
ii. pH-sensitive liposomes;
iii. Cationic liposomes;
iv. Immunoliposomes;
v. Long-circulating liposomes.

Sac size could be a decisive constraint in determining the circulation half-life of liposomes.

7.3.2 Classification of Liposomes by Size and Range of Bilayers

Liposomes are classified by their size and range of bilayers as follows:

i. Minute unilamellar vesicles (MUV): 20–100 nm;
ii. Large unilamellar vesicles (LUV): > a hundred nm;
iii. Giant unilamellar vesicles (GUV): > one thousand nm;

Table.7.2 Liposomes herbal formulations [12].

Herbal Sources	Phytoconstituents	Uses	% Entrapment Efficiency
Green tea, Apples, Berries, *Ginkgo biloba*, St. John's wort	Quercetin	Antioxidant Anticancer	67%
Milk Thistle	Silymarin	Hepatoprotective	70%
Sweet Wormwood	Artemisin	Treatment of viral infection	62–75%
Amur Peppervine	Ampelopsin	Antineoplastic	63%
Yew tree	Taxol	Antineoplastic	95%
Turmeric	Curcumin	Antineoplastic	88–89%

iv. Oligolamellar sac (OLV): 100–500 nm;
v. Multilamellar vesicles (MLV): > five hundred nm.

Attributable to their distinctive features, liposomes are capable of increasing the efficacy of different herbal extracts or phytoconstituents with mounting constituent liquefaction, rising element absorption, and by also increasing the cellular uptake of herbal drug. Liposomes release the active constituents of herbal drug to their site of action or their own receptor for long duration and this process will increase the safety of herbal drugs [26–28].

Silybin is a potent hepatoprotective herbal medicine which is isolated from the plant *Silybum marianum,* but oral absorption of silybin is very poor (< 30%) as compared to parental root. Formulation of silybin liposome increases the absorption more than 60% (26).

Various herbal extracts and phytoconstituents containing liposomal formulation are summarized in Table 7.2.

7.4 Nanoparticles

Nanoparticles are micron or submicron particles having a size less than 100 nanometers. The drug is dissolved, entrapped, encapsulated or connected to a nanoparticle matrix [29]. Nanocapsules are submicroscopic colloidal drug carrier systems composed of an oily or an aqueous core surrounded by a thin polymer membrane, whereas nanospheres are matrix systems within which the drug is actually and homogeneously spread. Recently, formulation of PEG nanoparticles has been used for significant delivery of therapeutic durgs because these nanoparticles will release the drug for a long

Table 7.3 Bioactive constituents and their applications [35–47].

Name of Bioactive Constituents	Application
Taxol	Decrease in poisonous effects
Curcumin	Boosted dissolution
Cuscuta Extract	Enhanced solubility
Triptolide Solid lipid nanoparticles	Enhanced bioavailability and anti-inflammatory activity
β-elemene	Improved constancy and bioavailability
Meletin	Improved drug discharge and antioxidant effect.
Silymarin	Enhance distribution time
Ammonium glycyrrhizinate	Enhanced absorption
Berberine	Persistent discharge of drug
Naringenin	Persistent discharge of drug and Enhanced bioavailability

period of time. PEG nanoparticles are also able to deliver DNA in gene therapy [30]. Some herbal nanoparticle constituents having a size of more than 100 nanometers have conjointly been rumored in the literature, like nanoparticles of curcuminoids having size of 450 nm, paclitaxel and praziquantel nanoparticles in size ranges from 150 to 200 nm. Additionally, nanoparticles may even be outlined as being submicronic (b1 lm) mixture systems [31]. Nanonization possesses several benefits, like mounting drug solubility, reducing meditative quantity of drug and raising the permeability of herbal constituents or extract compared with several classical herbal medication preparations [12]. Paclitaxel is an anticancer herbal compound which is isolated from the Pacific yew tree, *Taxus brevifolia.* Nanoparticles of paclitaxel delivered in a sustained release form reduce various poisonous effects. Curcumin, which is an active marker component of *Curcuma longa,* has malignant neoplasm activity but its anticancer use is very limited because of it having less solubility in water and poor absorbability. The nanoparticle formulation of curcumin has improved its bioavailability and has shown significant anticancer activity [32].

Prabhu *et al.* prepared silver phyto nanoparticles of Tulsi leaf extract and extract of nirgundi and compared their antibacterial drug activity to herbal extracts. They found that the antibacterial drug activities of all the herbal nanoparticles obtained from *Ocimum sanctum* showed the most maximum inhibitory rate using 150 μg of those plants extract [32].

Yamini *et al.* Synthesis of Silver Nanoparticles from *Cleome viscosa* and found that the extract of *Cleome viscose* was capable of manufacturing silver nanoparticles extracellular and are relatively constant in resolution it had been conjointly confirmed that the composite discharge of silver at a core is capable of interpretation antimicrobial affectivity and tried to move against the microbes [33].

7.4.1 Merits of Nanoparticles as Drug Delivery Systems

The diverse merits of using nanoparticles as drug delivery systems are:

i. Shelf life of herbal constituents will increase;
ii. Solubility of active constituents is markedly increased;
iii. Side effects related to dose of herbal extract is decreased;
iv. Nanoparticles target sites for action;
v. Water and lipid soluble constituents are delivered by nanoparticles.

7.5 Nanoemulsions/Microemulsions

Nanoemulsions are oil in water (o/w) emulsions having globule diameters starting at 50–1000 nm. Typically, the characteristic drop range is from 100 to 500 nanometers. In nanoemulsion, drug will exist as oil-in-water and water-in-oil forms depending on whether the central part of the drug is to be oil or otherwise water, correspondingly. Surfactants used for the preparation of nanoemulsions are approved for human consumption and routine food materials. These surfactants must be approved by the FDA in form of "Generally Recognized as Safe" (GRAS). Nanoemulsions are simply produced in large amounts by combining part aqueous insoluble oil with part aqueous compound under elevated shear pressure or motorized extrusion technique, which is available worldwide [48]. The different phases of nanoemulsions are shown in Figure 7.3.

Due to their smaller globule size and large surface area, nanoemulsions will easily absorb into the skin, so they should not have any type of virulent or irritant effects on the skin membranes. These formulations are also capable of transferring plant constituents to cell culture for treatment of cancer [48]. The drug delivery of curcumin by microemulsion increased its absorption as compared to simple curcumin emulsion [49]. For increasing the solubility and absorption of ubiquinone, a nanoemulsion of ubiquinone was also developed [50].

7.5.1 Merits of Nanoemulsions

There are various merits to using nanoemulsions, which are:

i. The extremely small drop size causes an oversized reduction within the gravity strength and movement might also be adequate for defeating gravity. This proposes that no physical occurrence happens on storage.

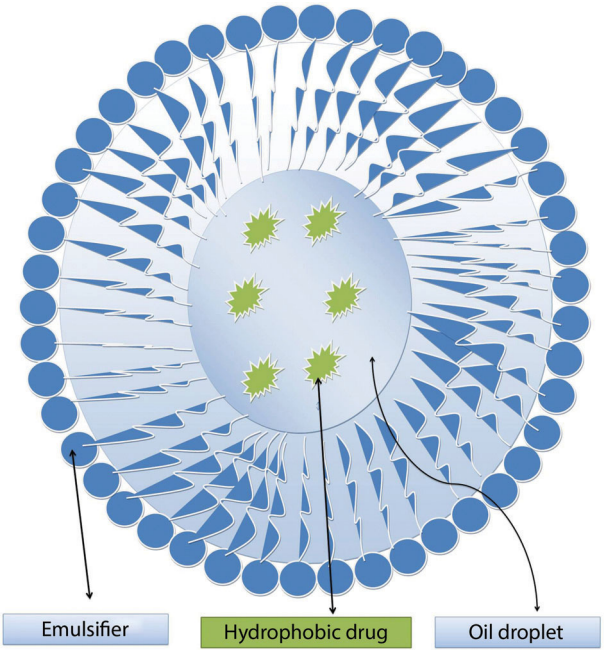

Figure 7.3 Different phases of nanoemulsion.

ii. The tiny drop size additionally avoids aggregations of the globule. Meager aggregations are prevented and this allows the system to stay spread with no separation.
iii. They are appropriate for well-organized delivery of active ingredients from the skin. The big expanse of the emulsion method permits speedy dispersion of drug molecules.
iv. Their clear character because of their liquidity (at affordable oil concentrations) and also because of the the nonappearance of thick particle nanoemulsions, which could provide a pleasing visual nature.
v. Unlike microemulsions, which need a high amount of surfactant to be more than 20%, nanoemulsions can be formulated by very minute amounts of surfactants. Formulation of 20% o/w nanoemulsion required less than 10% of surfactant.
vi. They are useful for the release of aromatic compounds, which can be included in several products; and are also useful for delivery of nonalcoholic fragrances.

7.6 Microspheres

Microspheres are typically free-flowing, elegant fine particles containing non-natural polymers that are fragile in character and preferably contain element dimensions smaller than 200 μm. Microspheres have a number of advantages to overcome the limitations of standard medical aid and increase the curative efficiency of herbal drugs [53] (Table 7.4). Numerous methods are available for delivering a drug to its target receptor

Table 7.4 Herbal microspheres and their applications [54,56–58].

Herbal Microspheres	Phytoconstituents	Advantages	Uses
Rutin alginate chitosan microcapsules	Rutin	Targeting into cardiocascular and cerebrovascular region	Cardiovascular and Cerebrovascular diseases
White turmeric oil microsphere	Essential oil	Continued discharge and elevated bioavailability	Liver protective
Camptothecin loaded microspheres	Camptothecin	Continued discharge of camptothecin	Treatment of cancer
Quercetin microspheres	Quercetin	Significantly decreases the dose size	Anticancer

or organ in a controlled release manner. Microspheres are one of the methods which release drug/extract in a systematic controlled manner to a specific target site in which the concentration of drug is also maintained during the treatment period without any side effects. Microspheres are not only popular for expanded discharge of drugs, but additionally for effective treatment of cancer by targeting the site of cancer cells [54]. Table 7.4 lists some herbal micropsheres and their applications.

Microspheres are smaller in particle size and have a large surface-to-volume magnitude relation. The surface possessions of microspheres usually increased the therapeutic activity [13]. Impervious microsphere and magnetic microsphere have been commonly formulated in the last few years. For the formulation of impervious microsphere the antigen and antibody are coated on the polymer microspheres. The microspheres are available in oral and parental dosage form.

Many phytoconstituents are formulated into microspheres like rutin, camptothecin, curcumin, quinine, quercetine and various herbal extracts [54]. Gastro suspended microspheres of silymarin are reported for continued controlled release of sylmarin for a period of 12 hours that showed an increased bioavailability of silymarin as compared to normal extract [55].

Microspheres are generally prepared by emulsion solvent evaporation, spray drying and chemical crosslinking techniques.

7.6.1 Classifications of Polymers Used in Microspheres

Polymers which are typically used in microspheres are classified into two types:

1. Synthetic polymers, which are classified into two types:

a. Non-perishable Polymers: Poly methyl radical methacrylate (PMMA), Acrolein, Glycidyl methacrylate, Epoxy polymers
b. Perishable Polymers: Poly alkyl group cyano acrylates, Poly anhydrides, Lactides, Glycolides and their copolymers

2. Non-synthetic polymers, which are acquired from entirely diverse resources like carbohydrates, proteins and with chemically changed carbohydrates [59].

7.7 Microcapsules

Microencapsulation is a method in which small elements or globules are encircled with coating to produce tiny capsules. Simply stated, microcapsules contain tiny globules which are covered by a homogeneous thin layer. In microcapsules the drug particle is cited as the middle part, inner part, or fill, whereas the layer is typically referred to as the case or surface covering. The dimensions of microcapsule range from 1 to 1000 micrometers [60,61] The herbal microcapsules entrapped with water-soluble extracts of plantain *Plantago major* and *Calendula officinalis L.* (PCE) were organized by layer-by-layer surface assimilation of gum and oligo chitosan onto carbonate microparticles with their sequent dissolving once EDTA treated. Covering of PCE was performed by absorption and coprecipitation techniques. The coprecipitation provided higher defense of PCE in the carbonate matrix compared to surface assimilation. *In-vitro* release kinetics were studied by simulated digestive juice. With the model of acetate lesions in rats, it has been verified that PCE free from the microcapsules accelerates stomachic tissue repair [62].

7.7.1 Morphological Features of Microcapsules [63]

The central material is key feature responsible for morphological changes of microcapsule (Figure 7.4).

1. Mononucleate (middle shell) microcapsules enclose the shell round the nucleus.
2. Polynuclear microcapsules contain numerous core capsulates inside the covering.
3. Matrix microcapsules in which the center substance is dispersed consistently into the covering matter.

7.8 Nanocrystals

Nanocrystals are nanoscopic crystals of the parent compound with dimensions less than 1μm. Formation of crystalline nanoparticles or nanocrystals improve solubility and porousness, and ultimately bioavailability [64] (see Figure 7.5). The solubility of phytopharmaceutical was increased even in nonsoluble product by its conversion into nanocrystal. The small element dimension means larger surface space which provides

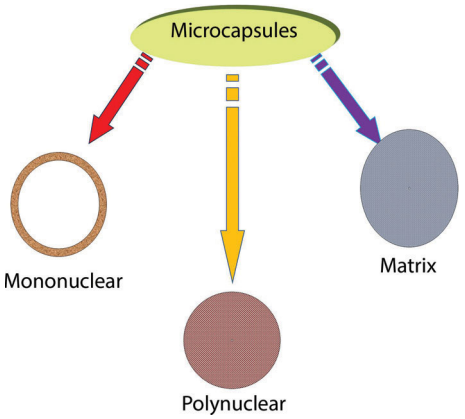

Figure 7.4 Morphology of microcapsules.

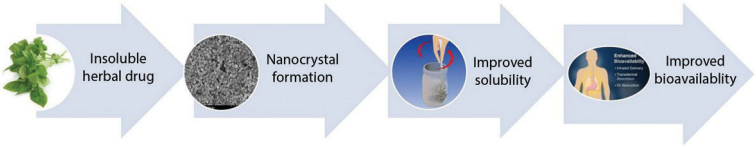

Figure 7.5 Nanocrystals of active herbal components.

access to a lot of solvent molecule that will encircle the elements, which is necessary for liquefying a drug molecule [65].

There are lots of phytopharmaceutical compounds available for use in the treatment of a variety of diseases. Gymnemic acids nanocrystals over microcrystals should overcome the absorption problem in the gastrointestinal tract and increase the bioavailability. The nanocrystals of gymnemic acids may offer necessary hints on how to move the medical ends of the herb to a novel pharmaceutical system for additional exploration for treatment of disease [66]. Ravichandran *et al.* prepared spray-dried curcumin nanocrystals by high homogenization technique that is used to supply the drug in capsule form. Dried drug nanocrystals provide superior chemical science properties. Curcumin nanocrystal-loaded capsules are often made via standard procedures. From the Noyes-Whitney equation, the hyperbolic area and saturation solubility attributable to the decrease in radius lead to hyperbolic dissolution velocity [67].

7.8.1 Methods for Formulation of Nanocrystals

The most common strategies utilized designed for the formulation of nanocrystals are crushing, aggressive blending and precipitation. Exhaustive analysis for novel tools has led to several different techniques meant for manufacturing of drug nanocrystals. Still, non-pharmaceutical firms like Dow Chemical have been getting into the market of weakly soluble medication with solubility-enhancing technologies.

Among different technologies, the subsequent critical fluid strategies are mentioned solely for reasons of completeness.

1. Rapid expansion of supercritical solution (RESS);
2. Rapid expansion from supercritical to aqueous solution (RESAS);
3. Solution-enhanced dispersion by the supercritical fluids (SEDS);
4. Spray freezing into liquid (SFL) evaporative precipitation into aqueous solution (EPAS); and
5. Aerosol solvent extraction (ASES) [68,69].

7.9 Ethosomes

Ethosomes were initially developed by Touitou *et al.* in 1997. Ethosomes are lipid vesicles usually known as ethanol modified liposome at concentration range b/w 30% v/*v* to 35% *v/v.* They have been widely used as topical/transdermal delivery systems effective for delivery of medication into and across the skin [70]. The high concentration of alcohol makes ethosomes a distinctive flexible carrier system which causes fluidization of the lipidic portion of skin. On its incorporation in an optimum amount into a membrane, i.e., vesicle boundry, it enhances the vesicle's ability to penetrate the stratum layer. Contrary to standard liposomes, ethosomes are generally smaller in size, higher in entrapment potency and, of course, have enhanced stability [71]. Ethosomes act as reservoir systems and offer continual delivery of medication to the desired site [72]. Examination of ethosomes by transmission electron microscopy reveals that ethosomes can either be unilamellar or multilamellar through to the core. Ethosomal vesicles may vary in size from a few nanometers to micrometers depending on factors and process parameters like method of preparation, stirring time, stirring speed and sonication. Divergent from transfersomes, ethosomes have shown improved skin delivery of drug in both occlusive and non-occlusive conditions [73]. The topical/transdermal routes have many advantages as compared to oral delivery, viz. patient compliance, ease of administration and removal, non-invasive delivery, direct systematic action, alleviation of first-pass metabolism, alleviation of GIT irritation and undesired metabolism, etc. [75].

The physicochemical properties of ethosomes enable this vesicular delivery system to move active moieties effectively through the skin in terms of amount and depth compared to standard liposomes that are remarkable primarily for delivering medication to the external layers of skin [75]. Triptolide-encapsulated ethosomes have been prepared for topical delivery of triptolide and evaluated for their ability to decrease erythema in rats as compared to the other formulations. Also, ethosomal formulation showed a rise in bioavailability, which may be because of increased skin accumulation. Ethosomes of *Sophora alopencerides* were prepared by transmembrane pH gradient technique. This ethosomal formulation has been proved as an extended release dosage form with increased permeability across the stratun corenum, and is thus used for topical delivery of alkaloids [76]. Ethosomal preparations of ammonia glycyrrhizinate for the treatment of inflammatory diseases of skin are also reported in the literature. These being less toxic and non-irritating, exhibited better bioavailability as compared to the ethanolic solution of the drug [77].

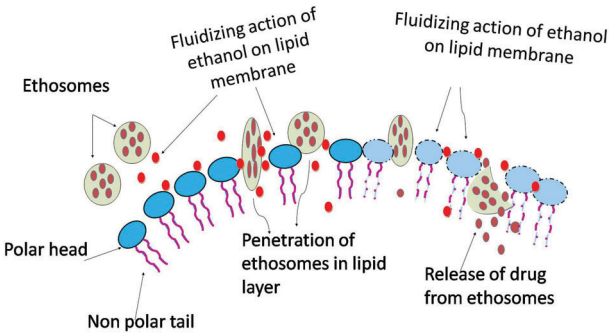

Figure 7.6 Planned mechanism for skin delivery of ethosomal systems.

Figure 7.6 is a diagrammatic representation of the mechanism of ethosomes-enhanced penetration of drug through lipids in stratum corneum. The layers of skin at body temperature are densely packed and enormously structured. Ethanol interacts with lipid molecules within the polar head cluster region, leading to an increase in their texture. The time interval for ethanol into the polar head cluster surroundings may end up in a rise within the membrane permeability. Ethanol also offers flexibility to vesicles, which enhances their penetration into deeper layers of the skin. The interdigitated, acquiescent ethosome vesicles will find their way through the disordered corneum.

7.10 Transfersomes

The word transfersome and the underlying idea were introduced in 1991 by Gregor Cevc. In the broadest sense, a transfersome can be an extremely adaptable and stress-responsive, complicated combination. The most popular kind of transfersome is ultra-deformable vesicle, usually having an aqueous compound core enclosed in a complicated lipidic bilayer. Reciprocity of the native composition and the form of the bilayer makes it both self-acting and self-optimizing [78].

Transfersomes were developed so as to uplift the transcutaneous drug hauler property of the phospholipid vesicles. These self-aggregated lumps, with the immoderate versatile covering, can repeatedly deliver the drug with great potency, either into or through the skin. However the effect may depend on the route of administration or mode of application. These vesicles have manifold improved skin penetration because of numerous orders of enormity higher elasticity than the quality liposomes formulation. Transfersomes, unlike ethosomes, overcome the skin penetration problem by compressing themselves on the intracellular protection lipid of the corneum and finding their way as flexible carrier between the lipids of the membrane [79].

7.10.1 Relevant Characteristics of Transferosomes

The infrastructure of transfersomes is composed of hydrophobic and deliquescent entrapped/embedded moieties. As a result, it has the capability to lodge active moieties

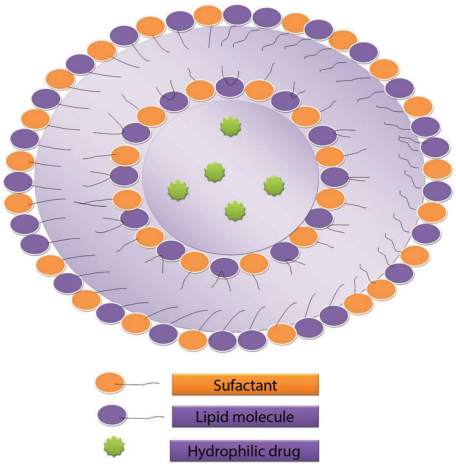

Figure 7.7 Surface of transferosome.

with a large range of solubility, as shown in Figure 7.7. The relevant characteristics of transferosomes are:

1. They deform and go through slender constriction (from five to ten times of their own diameter) with no measurable loss. This high deformability provides higher penetration of intact vesicles;
2. They act as carrier for a diverse range of molecules, e.g., analgesic, anesthetic, corticosteroids, endocrine, anticarcinogenics, insulin, albumen, etc.;
3. They have similar advantages to liposomes, which include biocompatibility, and are perishable as they are made up of natural phospholipids, soya phophatidylcholine [80].

7.10.2 Transferosomes as Herbal Formulation

Transferosomes encapsulating capsaicins, prepared by the high shear scattering technique, were found to have better penetration and high absorption in skin surface [81]. Curcumin is potentially used for the treatment of a variety of diseases, but it shows lower bioavailability when it is in the preferred oral dosage form. Formulation of transferosome curcumin gel increases the permeation of curcumin through the skin as compared to normal cucumin gel alone [82]. Transferosomes of periwinkle plant derivative vincristine sulpate showed higher permeation rate through the skin as compared to normal extract [83]. Colchicines, which are isolated from *Colchicum luteum,* have been used for the treatment of gout, but its oral absorption shows some serious gastric side effects, and the transferosme formulation of colchicines reduced its side effects and improved its bioavailability [84].

7.10.3 Limitations of Transfersomes

The limitations of transfersomes are:

1. They are prone to chemical degradation owing to their tendency of aerophilous degradation;
2. There is doubt that the purity of natural phospholipids can be estimated correctly, therefore, the world is against use of such drug delivery vehicles;
3. These formulations are uneconomical as well [80].

7.11 Nanoscale Herbal Decoction

The decoction of conventional Chinese medication containing Rhubarb stem, Huang Lian, (Coptidis stem) and skullcap is widely employed in the treatment of hepatic disorder [85]. However, decoction requires heating that lessens its effectiveness. Nanoscale San-Huang-Xie-Xin-Tang formulation contains Rhubarb stem, Huang Lian, (Coptidis stem) and skullcap with a weight ratio of 2:1:1, respectively. Nanoscale decoction of this herbal formulation was prepared in normal water, which potentiates the antihepatotoxic activity and bioavailability [86].

7.12 Natural Polymers in Nanodrug Delivery

Polymers, especially natural polymers, are one of the permanent candidates for preparation of drug delivery systems. They play an important role in the production of drug release devices and also in controlled drug release. The demand for a compound with specific physical-biological properties has created continued interest in the screening of natural polymers and their derivatives obtained from industrial and commercial sources [86]. Nano-drug delivery involves the use of nanometric carriers to deliver site-specific drug molecules. The nano size enables enhanced uptake of particles by cells in the body, resulting in greater retention by target tissues. The particles, ranging from 10 nm – 100 nm, facilitate the passage across cell membrane and reduce the potential for uptake and clearance by the reticulo-endothelial system, the liver and spleen. This causes increased therapeutic efficacy and a subsequent decrease in the

Table 7.5 Recent patents on novel herbal formulations.

Us Patent No.	Phytoconstituents	Novel Drug Delivery
US 5948414	Opium alkaloids and aloin	Nasal spray [92]
US 6340478 B1	Ginsenosides	Microencapsulation and sustained discharge [93]
US6890561 B1	Isoflavone-enriched fraction	Microencapsulation [94]
US0042062 A1	Extract of Soybin, turmeric and Ginger	Sustained discharge of active constituents for treatment of diabetes [95]
US7569236132	Meletin and Ginkgolide	Microgranules [96]

Table 7.6 Marketed novel herbal formulations.

Product Name	Plant Source	Novel Drug Delivery System	Company Name
White tea liposome Herbasec	*Tea leaves* extract	Liposome	Cosmetochem [97]
Green tea liposome Herbasec	*Tea leaves* extract	Liposome	Cosmetochem [98]
Ginselect Phytosome	*Panax ginsengrhizome*	Phytosome	Indena [99]
PA2 Phytosome	Bark of *Aesculus hippocastanum*	Phytosome	Indena [99]
Sericoside Phytosome	Root of *Terminalia sericeabark*	Phytosome	Indena [99]

facet of poisonous effects [87]. In addition, nanosizing leads to particles with an outsized surface area to volume quantitative relation, making a lot of space for particles to be adsorbed or carried [88]. Natural polymers are of great importance as a result of their usually being biocompatible, biodegradable, nontoxic and non-immunogenic. Naturally occurring polymers used can be classified into two groups, viz. polysaccharides and proteins [89].

Commonly used polysaccharide polymers for preparation of nanocarriers include starch, chitosan, alginate and dextran, while gelatin and albumin are examples of the proteins usually used [90]. These polymers are used to prepare colloidal particles of nanometric size. In these systems, the drug to be delivered might be spread among the compound matrix or adsorbed on the surface of the carrier, which is known as a nanosphere; or it might be encapsulated among a core surrounded by compound membrane, which is known as a nanocapsule. The tactic by which they are loaded into nanocarriers for delivery of drug is mainly due to their physicochemical properties, and therefore the drug to be loaded [91].

7.13 Future Prospects

Far-reaching research has been, and is still going on, in the area of targeted NDDS of active constituents and extracts obtained from plants. However, some aspects, viz. production, application and safety, are still at the exploration stage. The development of herbal remedies as novel drug delivery system (e.g., phytosomes, transferosomes, etc.) is still moving at an accelerated rate at a number of institutes at vital and clinical trial levels. However, the only prerequisite is to widen and utlize a formulation that not merely is as or more effective than the existing treatments for ailments, but that is also better at alleviating the side effects and increasing the patient's compliance. Hence, using

"herbal medication" in the form of nanocarriers could uplift the plant potential used for management of a range of persistent physiological disturbances. Nanocarriers as "herbal remedies" are also flourishing sources of many important medicinal compounds that can be used as purposeful foods. Several plant extracts and phytomolecules, despite having excellent bio-commotion *in vitro,* demonstrate less or no *in-vivo* actions due to their hydrophillicity or improper molecular size or both, resulting in deprived absorption and bioavailability. The incorporation of consistent plant extracts and polar phyto-constituents like terpenoids, flavonoids, tannins and xanthones in a novel formulation leads to enhanced absorption profile and higher concentration, reaching systemic circulation; thus resulting in improved pharmacokinetics of drugs. It is projected that the competent and vital importance of the natural products and herbal remedies proposed to be formulated as nano/microcarriers will augment the implication of existing dosage systems/forms used for herbal delivery.

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Endogenous Polymers as Biomaterials for Nanoparticulate Gene Therapy

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Abstract
Biomaterials with specific abilities to interact with biological structures have been widely used in the development of different devices intended for biomedical use. Within this field, the interest in endogenous polymers as components of delivery systems has gained increasing attention. This chapter aims to provide an overview of the use of these polymers in nanoparticle development and, more specifically, to present novel strategies that could open new horizons for gene therapy based on non-viral vectors. The strategies that we describe could offer a unique opportunity to support the translation of basic-science breakthroughs in endogenous polymers into clinical applications, thus offering the possibility to break down barriers in the transition from resource-based approaches towards knowledge-based strategies.

Keywords: Gene therapy, non-viral vectors, drug delivery, nanoparticles, endogenous polymers, proteins, carbohydrates

8.1 Introduction

The search for new drugs and therapeutic tools for the treatment of diseases is a continuous challenge in the pharmaceutical field. Among them, the gene therapy approach occupies a very promising place. The possibility of inducing the expression of a therapeutic protein (inserting a functional gene), or suppressing the aberrant expression of a protein (inhibiting the gene expression), opens countless possibilities to revolutionize the clinical practice [1–4]. However, this great potential future faces important issues that must be overcome if we want the broad clinical application of gene therapy to pass from hope to reality [5]. The first one is knowledge of the exact role of each protein in the physiopathology of a disease. Such information is necessary in order to

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predict if modification of the gene expression will result in any clinical improvement. This is a great challenge for multifactorial diseases—those where more than one gene is involved—and easier for diseases caused by only a single defective gene. The second issue is related to the design of a system able to protect and transport bioactive nucleic acids to the site of action without representing any risk to the patient. In this respect, different vehicles are being studied as carriers intended to provide successful gene transfer to cells [6,7].

Fundamentally, gene delivery systems can be differentiated into viral carriers (adenovirus, lentivirus, etc.) and non-viral vectors (complexes, micelles, liposomes, nanoparticles, etc.) [8]. Following are the major requirements for both polymers and nanoparticulate systems intended for gene therapy.

- Polymer:
  - *Biocompatibility and biodegradability* – the polymer should be metabolized by tissues and cells and the degradation products should be compatible with the body's structures.
  - *Absence of toxicity* – the polymer should present neither acute nor chronic toxicity. It should not elicit immune responses.
  - *Ability to form nanoparticles* – the polymer should be able to form a stable nanoparticulate system.
  - *Availability and affordability* – the polymer should be easily obtained at pharmaceutical grade and preferably at low price.
- Nanoparticle:
  - *Genetic material protection* – the system should be able to protect the therapeutic nucleic acid from degradation (extracellular nucleases, lysosomes, etc.).
  - *Specific cell uptake* – the system should be specifically internalized by the target cells to avoid potential off-target effects.
  - *Kinetic release* – the kinetic release should avoid early release of the nucleic acid but it should have an appropriate on-target release.
  - *Stability* – the system must show shelf stability as well as stability after administration (no aggregation, precipitation or undesired dissociation phenomena).
  - *Affordable production* – the system should be produced in an easy, large scale and cost-effective way, preferably avoiding harsh conditions (organic solvents, high temperature, toxic chemicals, etc.).

Among non-viral systems and from a historical perspective, as a premise of material related sciences concerning the selection or design of components for the development of nanoparticles intended for gene therapy, bioinertness has been a major requirement. Bioinertness means that a material should not provoke any reaction from the body. Thus, in the literature we can find different inert nanosystems, such as those based on polyesters [9–11]. However, this former position was reconsidered after understanding that the use of bioactive molecules or biomacromolecules could improve the targeting towards specific tissues/organs and provide an added biological value, such as mucoadhesiveness, the ability to interact with specific receptors, the

ability to provide stability in biological fluids, the ability to increase drug efficiency and even to provide synergistic effects [12–15]. These aspects are discussed in the following sections in which we refer to endogenous polymers, i.e., those polymeric components that are found in the human body. As a consequence, in the last few years there has been increasing attention on the use of endogenous materials that would fulfill such requirements [16,17]. Herein we will focus our attention on these non-viral endogenous polymer-based nanoparticles.

8.2 Polymeric Nanoparticles in Gene Therapy: Main Characteristics of Currently Proposed Nanosystems Based on Endogenous Polymers

The literature describes different nanosystems intended for gene therapy. Among these nanosystems, those based on polymers and at the submicron level are usually denominated nanoparticles. However, first of all we should clarify the difference between the terms nanoparticles and nanocomplexes (or complexes in general), as we refer to both in this chapter. Complexes can be described stoichiometrically and are usually formed by simply mixing cationic polymers and the nucleic acids in question. In such a case, without the active nucleic acid compound we would merely have a polymeric dispersion. On the contrary, in the case of polymeric nanoparticles the formation of the polymeric structure does not require the concurrence of an active compound, resulting in what it is possible to denote as 'blank' nanosystems. Due to the obviously more intricate nature of the interactions between polymers in nanoparticles (that can vary from covalent bounds to physically crosslinked interactions), they are generally recognized to have a superior ability to protect the nucleic sequence. Indeed, in some nanocomplexes the nucleic acid can be displaced or dissociated due to competitive interactions of the polymers with biological components.

8.2.1 Strategies Based on Use of Endogenous Polymers as Biomaterials

The interaction of nanoparticulate carriers with biological media will depend not only on their particle size, but also on two critical aspects: their exposed surface charge and composition. As a consequence, when we decide to modulate such interactions by using endogenous polymers we should focus our attention on the surface disposition of such polymers and their direct influence on the surface properties of the system. Therefore, we have two alternative possibilities to carry out the aforementioned design. The first consists of using the endogenous polymers as building blocks of the nanoparticle structure in order to provide the desired biomimetism. The second possibility consists of covering the surface of a preformed nanoparticulate carrier in such a way that the endogenous polymer faces the biological media and exposes its features.

An additional key aspect is to determine if we can use a selected endogenous polymer in its natural state or if we need to modify it in order to improve its characteristics,

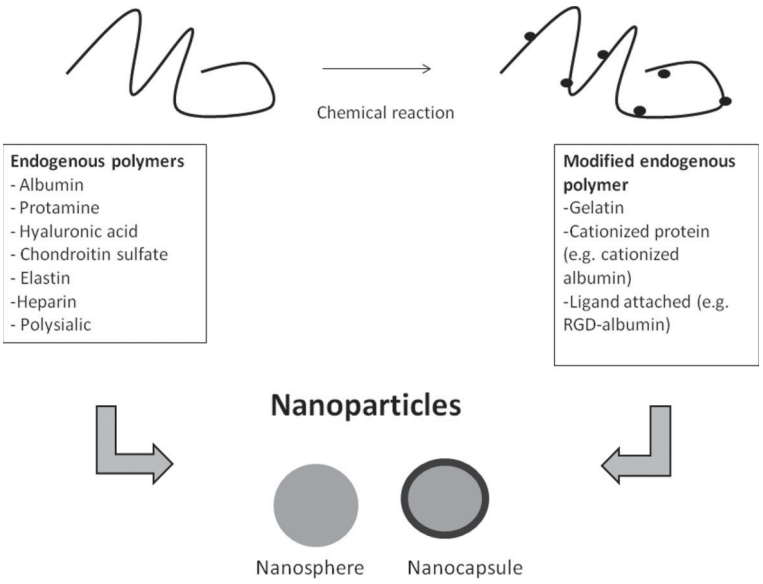

Figure 8.1 Use of endogenous polymers in nanoparticle formation: as endogenous polymers *in puris naturabilis,* as modified endogenous polymers or using combinations of both as well as with other synthetic/semi-synthetic polymers (hybrid nanoparticles).

possibilities that are illustrated in Figure 8.1. In the second case we can either modify it before nanoparticle formation (as raw material) or once the nanoparticle is already formed. Finally, some authors use modified endogenous polymers not due to their specific properties, but rather as the only solution to circumvent the limitations associated with their formulation as a nanoparticulate system. In the next sections we will discuss these possibilities.

8.2.1.1 Endogenous Polymers in Puris Naturabilis

Endogenous polymers can basically be classified according to their nature, either proteic or glycidic. Table 8.1 shows the most relevant endogenous polymers used for the development of nanoparticle-based gene delivery that will be discussed in this chapter. Proteins are more heterogeneous in their variety and they can establish complex interactions. Nevertheless, due to their tertiary conformation their structure is usually more rigid as compared to glycidic polymers. Besides, they are characterized by well-defined structures and very low polydispersity indexes. In contrast, carbohydrates are more flexible due to the characteristics of the glycosidic bounds (usually α-1,4) and present wide molecular weight distributions.

Although the inherent features of these endogenous polymers provide them with great potential for the design of different drug delivery approaches, their use in nanoparticle development is not an easy task. Most of these polymers are water soluble and do not behave well in organic solvents. Therefore, some preparation techniques widely used in nanotechnology are not suitable for the formation of nanoparticles using such compounds. Furthermore, the use of chemical crosslinkers, such as

Table 8.1 Main endogenous polymers used in the preparation of nanoparticles for gene delivery.

Class	Name	Highlights	Ref
Proteins	Protamine	Arginine-rich glycoprotein. Positively charged at physiological pH. Used to condense and protect nucleic acids. FDA approved as a drug.	[22]
	Albumin	The most abundant protein in plasma. Monomeric globular protein with high solubility. Component of the first FDA approved polymeric nanoparticle (Abraxene®).	[23]
	Collagen	Main structural protein. Composed mostly of glycine, proline and alanine residues. Triple helix structure. Hydrolysis leads to atelocollagen or gelatin.	[24–26]
	Elastin	Stimulus-responsive protein found in the connective tissue. Elastin-like polypeptides (ELPs) are recombinant polypeptides derived from elastin.	[27,28]
Carbohydrates	Heparin	Highly sulfated glycosaminoglycan composed of different disaccharides. Strongly interacts with antithrombin III and protamine. FDA approved as a drug.	[29–31]
	Chondroitin	Sulfated glycosaminoglycan composed of repeated units of N-acetylgalactosamine and glucuronic acid. Major component of cartilage and extracellular matrix.	[32–37]
	Hyaluronic acid	Non-sulfated glycosaminoglycan found in the connective tissue. Strong interaction with endocytic receptors (such as CD44). FDA approved as injectable cosmetic device (wrinkle filler).	[38–40]
	Polysialic acid	Homopolymer of N-acetylneuraminic acid. Advantages in tumor targeting and stealth properties.	[41–43]

glutaraldehyde, is not uncommon. They have the function of interconnecting and tightening up the components of the nanoparticle, while increasing the stability of the desired nanostructure. However, when using chemical crosslinkers it is necessary to verify that the nanoparticle preparation does not modify the natural properties and structure of the selected endogenous polymers before claiming any benefit of their incorporation on the basis of their inherent properties, a critical point that is too often overlooked.

Another important consideration when using endogenous polymers is the source of such polymers. Some of them, such as hyaluronic acid and albumin, can already be found as recombinant biotechnology products. However, some other polymers, such

as chondroitin sulfate, heparin and collagen, are mostly obtained from animal sources. This means that some safety issues must be considered, as well as some compatibility/immunological issues (especially relevant for polymers with a proteic nature).

Endogenous polymers can also be divided on the basis of their physicochemical characteristics at physiological pH, so they can be cationic or anionic polymers. Cationic compounds are usually required for non-viral gene therapy, since they can compact and protect the therapeutic sequence of genetic material. However, they are not easily found in the body. Important examples would be protamine and lysozyme, although only the former has been proposed as a biomaterial for gene delivery. Other endogenous low molecular weight amines have also been proposed in the formation of nanosystems based on natural compounds, but as ionic crosslinking agents and inducers of nucleic acids compaction. These amine compounds (e.g., spermine and spermidine) are present in almost all tissues and possess amine groups that are fully protonated at physiological pH. In this new approach, an inverse strategy to classic ionotropic nanoparticle preparation has been proposed based on negatively charged ionic crosslinkers such as tripolyphosphate or sodium sulfate [18]. The main interests in such a strategy are related to the possibility to: (i) develop nanoparticles based on anionic endogenous polymers such as hyaluronic or chondroitin without the necessity to modify them or use chemicals that can alter their high biological value (endogenous polymers *in puris naturabilis*); (ii) benefit from the specific properties of these biogenic polyamines, which are essential for tissue growth processes and cell differentiation and have specific receptors for their internalization. They possess special properties conferring protection to DNA against oxidative damage caused by oxygen reactive species (singlet oxygen and hydrogen peroxide) [19,20]. Increasing the intracellular levels of such polyamines can reverse aging-related processes, leading ultimately to increased lifespans in a great variety of life forms such as yeast, flies and human immune cells [21].

8.2.1.2 Chemical Modifications of Endogenous Polymers

The inherent characteristics of endogenous polymers may render them unsuitable to form nanostructures, provide nucleic acid protection or proper targeting. In such cases, chemical modifications may be required. Basically, they can be divided between those focused on improving nanoparticle formation or the association of active ingredients (such as modification using spermine) and those that can improve the cell targeting/internalization properties or the ability to interact with specific transmembrane receptors (e.g., modification using folic acid or sugars). As an example of the former, if we want to include albumin in a nanoparticle composition intended for gene therapy we should consider that its isoelectric point in water is 4.7. This means that at physiological pH (7.4) albumin is negatively charged, which would not allow the establishment of interactions with nucleic acids. However, cationization processes may provide a global positive charge to polymers that do not possess it naturally. Low molecular amines are generally used for such cationization processes, such as ethylenediamine, spermidine and spermine [44–48]. The reactions are normally carried out using carbodiimides, such as ethyl-3(3-diaminopropyl) carbodiimide (EDC) [49–53]. This allows amines to bind to the carboxylic groups of acidic residues in proteins. So not only is one potential negative charge blocked but the charge turns into a positive one. As a

result, modification using such compounds increases the isoelectric point of polymers and thus increases the affinity and strength of polymer-nucleic acid interactions. As an example, an increase in the isoelectric point of albumin allows the production of nanoparticles of cationized albumin associated with siRNA, such as those prepared by Han *et al.* (2013) (mean size 260 nm and positive zeta potential +30 mV) [54]. Nevertheless, it is important to notice that the addition of amine groups can drastically increase the toxicity of the resulting polymer at the same time, even for those with high biocompatibility in their natural state [55]. In addition, serum-induced aggregation of nanosystems based on cationized polymers such as albumin has been reported after IV administration, resulting in aggregates with a size up to 5 μm, leading them to be trapped in the lung capillaries, as has been previously reported for other cationic vectors [56,57]. Even though this lung preference can be used to design specific treatments, it should be considered a major concern due to potential undesirable damage associated with the described aggregation after parenteral administration. Other examples of undesirable effects can be found in the literature using other administration routes. Thus, after intravitreal injection of albumin-based anionic particles free diffusion in the posterior direction from the vitreous to the retina can be observed, but most cationic particles remain bound and aggregated in the vitreous [58,59].

To illustrate the aforementioned approaches, Figure 8.2 shows some ligands that have already been used in polymer modification for drug delivery systems and that therefore may find special applications in gene therapy.

The tripeptide RGD is a signaling domain derived from fribronectin and laminin and is composed of L-arginine, glycine and L-aspartic acid. This sequence is a common element in cellular recognition implicated in cellular attachment via integrins and is one of the most described approaches for the development of biomimetic biomaterials [60,61]. For instance, albumin nanoparticles grafted with RGD demonstrated 61-fold enhancement in receptor-specific mediated cellular delivery of oligonucleotides in integrin-expressing tumor cells compared to non-targeted control nanoconjugates. They

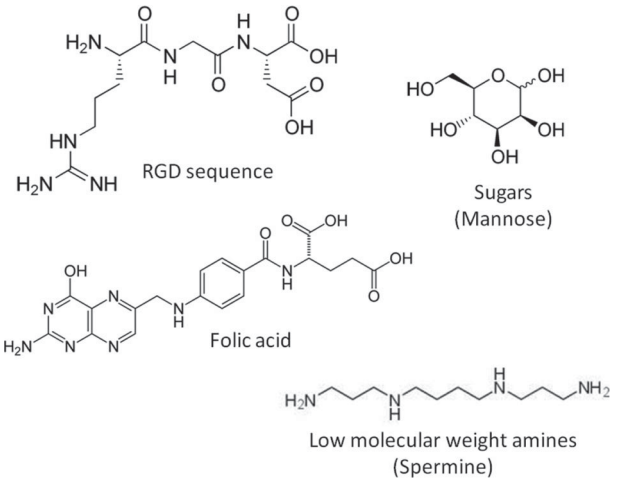

Figure 8.2 Examples of ligands used to provide new features to endogenous polymers.

were also able to enhance the functional activity of the oligonucleotide at low nanomolar concentrations without causing expressive cytotoxicity [62]. RGD sequences have also been used to modify the endogenous polymers collagen [63,64] and hyaluronic acid [65], improving their interactions with cells. It is important to highlight that although these modifications were not intended for nanoparticulate gene therapy, they open interesting possibilities for targeting specific cell groups.

Folic acid is a high affinity ligand of the folate receptor, which is overexpressed in some type of cancers such as ovarian or lung cancer but absent in normal cells. If derivatized by its γ-carboxyl group it keeps the receptor binding affinity [66]. Folate conjugation, therefore, presents an alternative method of targeting the folate receptor. This strategy has been successfully applied *in vitro* not only for gene therapy but also for receptor-specific delivery of anti-T-cell receptor antibodies, interleukin-2, chemotherapy agents, radiopharmaceuticals, and imaging contrast agents, among others [67]. Covalent conjugation of folic acid on the surface of albumin nanoparticles via carbodiimine reaction led to increased nanoparticle uptake by cancer cells (neuroblastoma cell line UKF-NB-3, and rat glioblastoma cell line 101/8) but not by normal cells (primary normal human foreskin fibroblasts) [68]. The same approach was also used for albumin nanoparticles targeting SKOV3 cells (human ovarian cancer cell line), being nanoparticles (~70 nm) successfully internalized by the cells by means of the folate pathway [69]. The covalent conjugation of folate onto the surface of the nanoparticles seems to be crucial in order to improve the nanoparticles' affinity for the receptor. Folate-adsorbed nanoparticles exhibit inferior affinity in comparison to covalent-bound ones [68].

As previously mentioned, chemical modification of polymers by the addition of sugars can also provide new features to endogenous polymers. Native carbohydrates containing aldehyde groups as reducing ends can be directly coupled with amine-containing molecules, leading to the formation of a Schiff base intermediate. Asialoglycoprotein receptors presented in the liver can be targeted by using polymers with lactose or galactose residues. Thus, galactosylated albumin-polyethylenimine nanoparticles tested *in vitro* for gene transfer showed cellular uptake specifically dependent on the abundance of galactose receptors on target cells in a cultured human hepatoblastoma cell line (Hep G2 cells) and mouse fibroblast cells (NIH/3T3 cells) [70]. Another interesting approach is modification of the polymer with mannose. In this case, the target is the mannose binding protein (MBP), a lectin-type protein that takes part in the innate immune response, being present in certain types of cells, e.g., hepatic and phagocytic cells (such as macrophages and dendritic cells) [71]. Pharmacokinetic studies revealed a greater internalization rate in comparison with other liver-specific carriers based on galactosylated, succinylated and cationized albumin. After 2 h of intravenous administration, 70% of mannosylated-bovine serum albumin accumulates in the liver, with Kupffer cells being responsible for 66% of this uptake [72]. Such a functionalization with mannose has also been successfully described for drug and gene delivery using other nanosystems such as liposomes and solid lipid nanoparticles [73–75].

To obtain a benefit from the properties of the aforementioned moieties while preserving the delicate nature and properties of the endogenous polymers, modification reactions should be mild and preferably take place in aqueous environment. Harsh conditions may result in hydrolysis, irreversible lost of endogenous polymer conformation

and/or inherent properties. Figure 8.3 exemplifies the most suitable reactions for the modification of endogenous polymers.

Carbodiimides are extensively employed for activation of carboxylate groups by the formation of highly reactive O-acylisourea intermediates (Figure 8.3a). This active species can then react with amine nucleophiles to form stable amide bonds. In the case of reductive amination (Figure 8.3b), the result is a linkage between the aldehyde and amine components, forming stable amine bonds without introducing any spacer. Maleic acid imides (maleimides) are also an integral part of many heterobifunctional crosslinking agents, allowing the covalent attachment of bioactive molecules to polymers in a two-step procedure (Figure 8.3c). Finally, isothiocyanates are homobifunctional linkers that react with primary amines leading to the formation of stable thiourea compounds (Figure 8. 3d) [76].

Although all the above described approaches may be very useful in drug delivery, it is necessary to be aware that modification of the native polymeric structure may dramatically alter the most interesting properties of the endogenous polymer, such as biocompatibility and toxicity and, in any case, the material that was once innocuous and FDA approved now is a completely different chemical entity and would likely face a long wait before its approval by any regulatory agency. Thus, the whole idea of using completely biological and endogenous materials is gone when using modified polymers. Conceptually, some of them are fine and elegant approaches but they lack practical feasibility. It is the task of the pharmaceutical researcher to consider all these variables and limits when designing any new drug delivery system.

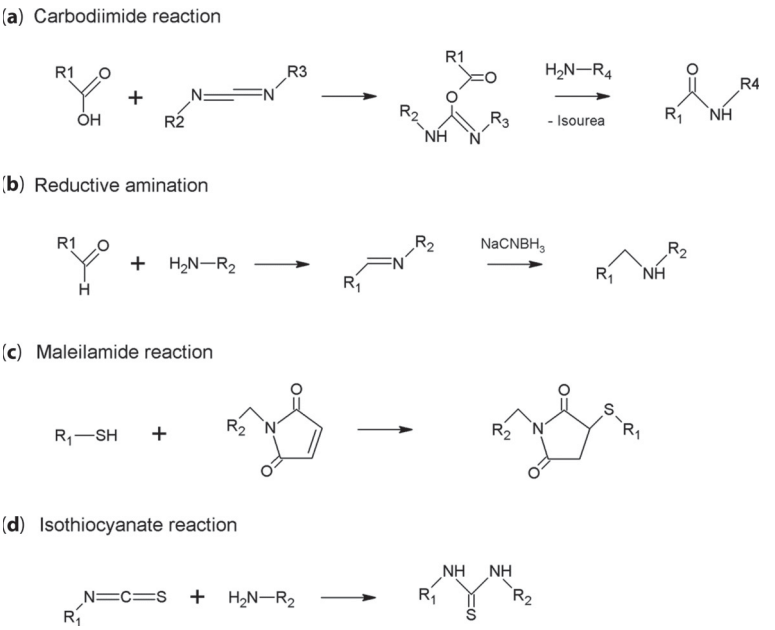

Figure 8.3 Main conjugation mechanisms used in polymer modification.
(a) Carbodiimide mediated reaction of amines with carbonic acids. (b) Reductive amination. (c) Reaction of maleimides with thiols. (d) Reaction of isothiocyanates with nucleophiles.

8.2.2 Physicochemical Characteristics of Nanosystems Based on Endogenous Polymers

Although a detailed description of the main physicochemical characterization techniques used in polymer characterization is clearly beyond the scope of this chapter, we have included specific details to be considered when such characterization is specifically for nanoparticles for the purpose of gene therapy. In such a situation we should take into consideration the following parameters and details:

8.2.2.1 Molecular Weight

The molecular weight of polymers is perhaps one of their most important physicochemical characteristics to take into account. In some cases, the biological activity of an endogenous polymer is molecular weight dependent as well as their ability to form nanostructures, as will be further discussed. Several techniques are available to determine the different ways of expressing the molecular weight parameter. The most relevant expressions for the development of nanoparticulate systems are the weight-average molecular weight (Mw), number-average molecular weight (Mn) and viscosity molecular weight (Mv). The polydispersity of polymer molecular weight (Ip) differs from the polydispersity index of the particle size (PdI). The former can be calculated from the ratio between the weight-average molecular weight and the number-average molecular weight, as described in the following equation:

$$\mathrm{Ip} = \mathrm{Mw}/\mathrm{Mn} \tag{8.1}$$

In the case of proteins, low polydispersity and well-defined values for the molecular weight are expected as compared with polysaccharides. On the other hand, the molecular weight distribution of polysaccharides can be strongly influenced by their source and method of obtention.

Ideally, for the formation of a nanoparticulate system by complexation/ionic gelation, the polymeric solution should be in one semi-diluted regimen, the optimal concentration being:

$$c = 1/[\eta] \tag{8.2}$$

where c is the concentration of the starting polymeric solution and $[\eta]$ is the intrinsic viscosity. This is an important issue since concentrated solutions often induce uncontrolled precipitation. If we relate this to the Mark-Houwink equation, we can see a relation between the intrinsic viscosity and the molecular weight of the polymer as follows:

$$[\eta] = KM_v^a \tag{8.3}$$

where M_v is the molecular weight viscosity and K and a are the Mark-Houwink constants that depend on the polymer-solvent system. So, a polymer with a higher molecular weight would theoretically require a more dilute solution for the proper formation

of nanoparticles (i.e., precipitation in the nanometric state). On the other hand, the use of small oligomers may be problematic since ionic crosslinking may not hold these oligomers together sufficiently to lead to the required 3D network of particles.

It is also important to keep in mind that the molecular weight has a strong correlation with the intrinsic toxicity of the material, especially if it has suffered any chemical modification. Higher molecular weight is usually associated with higher toxicity (as described for poly-L-arginine, one poly-amino acid widely used in gene therapy), but this profile must be tested in each new instance since, in some cases, lower molecular weight chains have resulted in higher toxicity.

The main techniques used to determine the molecular weights of polymers are analytic ultracentrifugation, osmometry, static light scattering and viscometry. Size exclusion chromatography with triple detection (refractive index/UV, capillary viscometer and static light scattering) gives the finest results, especially when a multi-angle light scattering detector is used. In the case of proteins, where the molecular weight is well known, acrylamide-gel electrophoresis can be used to confirm the structure of the polymeric chain.

8.2.2.2 Size

The size of a nanostructure may determine its *in vivo* behavior, activity and stability over time. Also, the internalization of nanostructures is a size-dependent process, as will be further discussed. The size, however, does not necessarily have a correlation with the molecular weight of the polymer. It is more dependent on the ratio of the constituents in the formulation and the conformation of the polymers used. More rigid structures, such as atelocollagen or albumin, may lead to particles of larger size. Dynamic light scattering and laser diffraction are the most relevant techniques for size determination. Dynamic light scattering, sometimes referred to as Quasi-Elastic Light Scattering, is a well-established technique for measuring the size and size distribution of molecules and particles, typically in the submicron region. Laser diffraction determines particle size distributions by measuring the angular variation in the intensity of light scattered as a consequence of the Tyndall effect as a laser beam passes through a dispersed particulate sample. Large particles scatter light at small angles relative to the laser beam and small particles scatter light at large angles. The angular scattering intensity data is then analyzed to calculate the size of the particles responsible for creating the scattering pattern, using the Mie theory of light scattering. The particle size is reported considering a sphere with same diffusion coefficient. Polydispersity index (PdI) is one parameter that is often associated with the average size of nanoparticles. It measures the width of the particle size distribution and is a mathematical description of variability in size. The formula that represents the PdI is:

$$\mathrm{PdI} = \frac{(\sigma)^2}{\mathrm{d}} \tag{8.4}$$

where σ is the standard deviation and d is the average diameter. It can range from 0 to 1, "1" being completely polydisperse and "0" completely monodisperse. Optimal values vary according to the use and technique employed in the preparation of the

structures, but values less than 0.2 can be considered monodisperse for most polymeric nanoparticles.

Microscopy techniques may also be used to perform size estimations and to corroborate the size homogeneity in a population. Transmission electronic microscopy (TEM), scanning electronic microscopy (SEM) and atomic force microscopy (AFM) are the most used. Nevertheless, these techniques are more often applied for visual characterization of size and distribution in order to corroborate data obtained with other techniques. This is because the theory behind most of the techniques used for size determination relies on the use of monodisperse and spherical particles. Any deviation from this condition may give unrealistic results that consequently may affect the efficacy of the system. In TEM measurements, a contrast agent is normally necessary for proper visualization, such as uranyl acetate, osmium tetroxide, ammonium molybdate or phosphotungstic acid. Because of its high resolution, AFM can also be useful to detect interactions between plasmids and nanostructures [77].

8.2.2.3 Electrical Properties

The zeta potential is the parameter used to characterize a nanostructure's surface charge. Although zeta potential is not measurable directly, it can be calculated using theoretical models applied to the data provided by experimentally determined electrophoretic mobility or dynamic electrophoretic mobility, electrokinetic phenomena and electroacoustic phenomena being the usual sources of data for the calculation of zeta potential. Keeping the zeta potential far from a neutral value is important in order to avoid stability problems in systems once electrostatic repulsion is favored. Indeed, zeta potentials close to neutral result in a tendency to aggregate, and a high zeta potential (in module) is usually associated with stable systems.

Ionic interactions are also used to associate genetic material with nanoparticulate carriers. Electrophoresis is perhaps the easiest way to assess the association efficiency of nucleic acids to a nanostructure. The associated genetic material will not migrate in an electrophoresis gel as would happen with free nucleic acids. It is also useful to visually determine the conformation of the plasmid DNA, supercoiled being the most effective form in comparison to circular and open forms. The association efficiency can be quantitatively determined using commercial kits and fluorescence techniques or, directly but with a lower sensitivity, by UV determination. For this purpose, the free nucleic acid needs to have been previously separated and subsequently quantified.

The nature of the zeta potential of the resulting DNA-nanoparticle structures (either positive or negative) may also be a determinant of the route of administration, toxicity or nucleic acid stability. A positive zeta potential seems to be very important for increasing the interactions between nanoparticles and the cell surface and is usually associated with more effective transfection systems. However, negative zeta potentials are required in order to avoid certain undesirable interactions with the biological environment that can lead to system instability and side effects.

8.2.2.4 Chemical Interactions

More complex interactions than electrostatic ones between the constituents of a nanostructure or between the polymer and nucleic acids require more sophisticated characterization techniques in order to understand the mechanisms underlying these interactions and the corresponding implications. In this respect, nuclear magnetic resonance spectroscopy (NMR) is a multipurpose, high-resolution and nondestructive analytical technique that can be used for both qualitative and quantitative characterization purposes. This technique is useful not only for the characterization of raw materials but can also be applied to obtaining qualitative and quantitative information about these same components in the nanoparticulate state, especially those concerning the interactions that take place [78,79]. Other techniques that provide useful information are Fourier Transform-Infra Red (FT-IR) spectroscopy, small angle X-ray (SAXS) and differential scanning calorimetry (DSC).

8.2.3 Nanoparticle Internalization

Nanoparticle internalization by the target cells is a prerequisite for the proper functioning of any gene therapy strategy based on the use of these systems. Such internalization can be mediated by different mechanisms, such as phagocytosis and pinocytosis. Phagocytosis is restricted to a number of specialized cells, such as macrophages and lymphocytes. Other cell groups usually take up nanoparticles by pinocytosis, which can be divided into macropinocytosis (>120 nm), clathrin-mediated endocytosis (~120 nm), caveolin-mediated endocytosis (~90 nm) and clathrin and caveolin-independent endocytosis. Although surface composition and charge have a clear impact on nanoparticle internalization, as described above, these mechanisms are also size dependent [80,81]. Thus, albumin nanoparticles with a mean size of 150 nm have been described as internalized by both clathrin and caveolin-mediated endocytosis [82].

After being successfully internalized, lysosomal escape is required in order to avoid degradation of the associated nucleic acid sequences. The mechanisms underlying such escape are yet to be completely understood. However, it is proposed that some cationic polymers can act like "proton sponges," as previously discussed, thus avoiding a rapid drop in intra-lysosomal pH as well as leading to lysosomal rupture by increasing the influx of water.

The internalization process can be favored by specific interactions between receptors on the surface of target cells and the endogenous polymers that form the nanoparticles, as described above. For instance, CD44 is a transmembrane glycoprotein known to be involved in the binding, endocytosis, and metabolism of hyaluronan, playing a significant role in cell adhesion, inflammation and immune response reactions. The cellular interaction strategy proposed for some nanostructures containing hyaluronic acid is illustrated in Figure 8.4 [83]. Another receptor that acts in a similar way is the hyaluronic acid receptor for endocytosis (HARE). It mediates the clearance of hyaluronic acid and chondroitin sulfate from lymph and blood in mammals. The affinity of HARE for chondroitin sulfate is about three times greater than for hyaluronic acid [35–37,40,84–87].

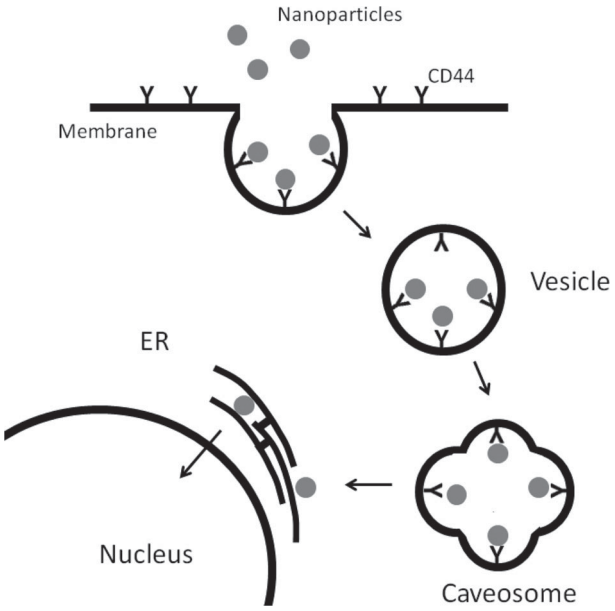

Figure 8.4 Proposed internalization pathway for hyaluronic acid-based nanoparticles loaded with plasmid. The nanostructures interact with CD44 receptors in the plasma membrane, triggering the internalization of caveola vesicles that then fuse with the caveosome. The plasmid is release from the nanoparticles and finally is able to enter the nucleus.

8.3 Specific Features of Endogenous Polymers that Can Open New Prospects in Nanoparticulate Gene Therapy

8.3.1 Proteins

8.3.1.1 Albumin

Human serum albumin (HSA) is the major plasma protein, with a globular structure and a molecular weight of 66.4 kDa. Its relatively good stability against heat (60°C) and pH (from 4 to 9), as well as low toxicity and immunogenicity, make albumin a very interesting material for drug delivery [88,89]. Furthermore, albumin exhibits a high capacity for binding drugs due to the presence of multiple binding sites. Indeed, many metabolic compounds and therapeutic drugs are transported by albumin. Most of the interest in albumin for gene therapy is based on its well-known ability to avoid undesired interactions with serum proteins, which often occur after intravenous injection of transfection systems [89].

Coating nanostructures with albumin may be useful to prevent their interaction with serum proteins and improve intracellular uptake. Thus, at proper concentrations albumin has the potential to minimize interactions of serum proteins with siRNA nanoparticles intended for effective systemic *in-vivo* siRNA delivery [90]. In addition, because of its negative charge, HSA may decrease the surface charge of LNPs and increase serum stability. As an example, an HSA coating on nanoparticles significantly

decreased florescence in phrGFP-transfected MCF-7 cells in knocking down experiments. In the presence of FBS (5%), the fluorescence after exposure to siRNA-loaded lipid nanoparticles coated with albumin significantly decreased phrGFP fluorescence at a HSA-to-siRNA ratio of 2:10. phrGFP was significantly down-regulated in phrGFP-transfected MCF-7, MDAMB-231, and SK-BR-3 cells only when albumin was used to coat the lipid nanoparticles [91]. An albumin coating has also been used to stabilize gold nanoparticles [92] and silica nanoparticles [93]. Citrate-coated gold nanoparticles rapidly aggregate and precipitate at their isoelectric point. However, zeta potential measurements proved that albumin-coated gold nanoparticles are stable at their isoelectric point. Both the albumin and the nanoparticles had nearly the same isoelectric point and were negatively charged at neutral pH, which provided electrostatic stabilization in addition to steric stabilization [92].

Concerning toxicity, in three human colon (CaCo-2), lung (A549) and macrophage (THP-1) cell lines as well as two lung (ASB-XVI) and colon (Colon-26) mouse cell lines, albumin-stabilized nanoparticles were considerably less toxic than the unmodified nanostructures [93], also showing no significant effect on the cell viability of mouse mammary tumor 4T1 cells [90].

Although gene manipulation techniques offer the possibility of making recombinant human serum albumin (rHSA) [94], numerous studies have used bovine serum albumin (BSA) in the development of drug delivery systems, more due to the economic factor than to the chemical differences between HSA and BSA. Both proteins have similar folding and a well-known primary structure; the most notable difference is the presence of two tryptophan residues in bovine albumin, while human albumin has a unique tryptophan [95].

The most common way to prepare albumin nanoparticles is by desolvation followed by chemical crosslinking, usually based on glutaraldehyde [82,96]. The amount of crosslinker used has been shown to influence the loading of plasmid DNA. The organic solvent, usually ethanol, is further removed by evaporation under reduced pressure [82,97]. Albumin nanoparticles were studied for local expression of tissue-type plasminogen activator (t-PA), a thrombolytic agent used to prevent thrombosis after mechanical heart valve replacement under therapeutic ultrasound. In a dog model of mechanical tricuspid valve replacement, t-PA expression was achieved over a prolonged period of time (up to 8 weeks) with no reduction in prothrombin time, which could lead to systemic side effects [97]. Albumin nanoparticles have also been used as efficient drug delivery systems for antisense-oligonucleotides (AS-ON) with anti-cytomegalovirus activity [96]. The nanoparticles induced a diffuse cytoplasmic distribution and major nuclear accumulation of AS-ON in MRc5 cells (fibroblasts). However, they offered only limited protection for the oligonucleotides against enzymatic degradation (60 minutes), a fact that may limit the use of this approach for *in-vivo* applications.

8.3.1.2 Collagen and Derivatives

Collagen is the main component of the extracellular matrix and is widely used as a biomaterial due to its promising biocompatibility, low antigenicity and biodegradability [98]. Although collagen forms hydrogels without the need for chemical crosslinking agents, due to their weak mechanical strength nanoparticle preparations need

additional treatments. For instance, collagen nanoparticles are often prepared by electrostatic interactions with sodium sulfate [99]. Even though collagen is a promising material for tissue engineering, it has led directly to only a few applications in the development of nanoparticulate gene carriers. On the contrary, promising results have been obtained with collagen derivatives such as atelocollagen and gelatin. Specifically, atelocollagen—a purified pepsin-treated type 1 collagen with molecular weight around 300 kDa—is one of the most used polymers for gene delivery to tumors, especially using siRNA [100]. In this case, the rigid structure of the atelocollagen can easily interact with the rod-like shape of the siRNA sequences. Intratumoral injections of atelocollagen-siRNA nanocomplexes downregulated FGF-4 expression in NEC8-luc-testes xenograft tumors in nude mice, reducing tumor growth at day 21 post-intratumoral injection [101]. They were also able to reduce expression of the HPV16 E7 oncoprotein, reducing tumor size [102]. Inhibition of metastasis in bone tumor was achieved by silencing EZH2 and P110-α after IV injection [103]. Similar results were obtained for liver cancer in A549-luc-liver metastases in a nude mice model after inhibition of PLK-1 [104]. However, the formation of atelocollagen nanoparticles is not favored due to its rigidity.

Gelatin is obtained from collagen by acid and alkaline hydrolysis, resulting in glycine, proline and 4-hydroxyproline residues with a typical structure of -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro. A number of methods have been reported to prepare gelatin-based nanoparticles including desolvation, a thermodynamically driven self-assembly process for polymeric materials, emulsion, chemical crosslinking, nanoprecipitation and coacervation [98,105]. Desolvation of gelatin solutions induces transitory coil-helix formation followed by aggregation of the helices due to the formation of a collagen-like triple-helix, enabling the formation of nanoparticles. Moreover, the large number of functional groups on the polymer backbone can be used for crosslinking purposes and the addition of ligands. However, nanoparticles formed on the basis of these characteristics are transient and require further stabilization. Chemical crosslinking with glutaraldehyde is one easy way of stabilizing these structures.

A negatively charged type B gelatin at neutral pH (7.0) can physically encapsulate reporter and therapeutic nucleic acid constructs, as opposed to positively charged lipids and polymers that electrostatically condense DNA [106]. However, the most promising results have been achieved using cationized gelatin. The cationization process is similar to the one already described for albumin, using low molecular weight amines and water-soluble carbodiimines. This cationization can be carried out either on the gelatin prior to nanoparticle formation (as raw material) or on the pre-formed gelatin nanoparticles. In the later case, ionic gelation/nanoprecipitation is the most often used preparation technique [89].

Nanostructures containing cationized gelatin are able to protect pDNA and siRNA from nuclease degradation and transfect a variety of cell types and tissues, both *in vitro* and *in vivo*. After cationization with ethylenediamine or spermine, the levels of transfection efficiency achieved can be compared to those provided by classic transfection agents such as polyethyleneimine. Table 8.2 shows the most important results obtained with nanoparticles based on cationized gelatin for gene delivery.

However, all the above-mentioned results were obtained using polymers from animal sources. This suggests that they will present important safety issues related to their immunogenicity and potential contamination (e.g., spongiform encephalopathies).

Table 8.2 Compilation of studies employing gelatin cationized with the amines cholamine (CA), ethylenediamine (ED) or spermine (SPM).

Amine	Type of Therapy	Observations	Ref.
CA	AS-ON	Strong immune system stimulation and activation of specific cell groups.	[107,108]
	pDNA	Lower toxicity than polyethylenimine complexes. Good reproducibility of the methodology with the potential to scale up.	[109]
	siRNA	Poor stability influenced by environmental pH and ionic strength. Freeze drying may improve the stability.	[110,111]
ED	pDNA	Ability of cationized gelatin to form nanoparticles by ionic gelation	[112]
SPM	pDNA	Hybrid nanoparticles with the anionic polymers chondroitin sulfate or dextran sulfate led to *in-vitro* and *in-vivo* ocular transfection with a therapeutic gene.	[113–115]

Recombinant DNA technology can help to solve this problem, as recombinant human gelatin (rHG) can already be found on the market.

8.3.1.3 Protamine

Protamines are small nuclear proteins that replace histones in the haploid phase of spermatogenesis. Because they are arginine-rich proteins they are positively charged at physiological pH, being able to interact with DNA to enable its condensation and stabilization. Protamine can be used in the form of the sulfate or free base, two different forms that may significantly influence the physicochemical properties of the nanoparticles, especially their size [116], the sulfate salt being the most used form of the polymer.

Protamine-based nanoparticles show a very low cytotoxicity in comparison with other gene delivery systems. In addition, when compared with commercially available liposomes (DOTAP, lipofectin), one artificial virus capsoid (polyoma VP1) and two cationic acrylate nanoparticles, the cell transfer efficiency in a mouse fibroblast cell line with protamine nanoparticles was one of the highest [117].

The most promising siRNA cell uptake results have been obtained using low molecular weight protamine. *In-vivo* studies with tumor-bearing mice further demonstrated that the peptide could carry and localize siRNA inside tumors and inhibit the expression of VEGF through systemic application of the peptide complex, thereby suppressing tumor growth [118]. Low molecular weight protamine possesses cell translocation activity and can be used to increase internalization in certain cell types since it has an internalization pathway similar to those found for TAT, the major internalization ligand for HIV. Several hypotheses have been suggested to describe how cell-penetrating

peptides mediate cell transduction by protamine, including the formation of hydrogen bonds between guanidine head groups in the arginine residues and phospholipids on the cell membrane, and the interaction of the arginine residues with extracellular matrix glycosaminoglycans such as heparin sulfate or chondroitin sulfate. Protamine/AS-ON nanoparticles were able to provide specific inhibition of TAT mediated HIV-1 transactivation, their uptake by macrophages being increased 25-fold in comparison to that obtained with free AS-ON [119].

In spite of these advantages, using protamine as a nanoparticle component presents some disadvantages, such as: (i) potential aggregation and precipitation phenomena in aqueous solutions under isotonic conditions and (ii) poor dissociation of nanoparticles captured intra-cellularly in the endosomes [120]. To overcome these problems, human serum albumin has been added to such complexes in order to inhibit precipitation of the particles. The albumin-protamine/AS-ON complexes showed an enhanced release of ON into the cytoplasm as compared to the binary system of AS-ON and protamine [120]. In addition, the combination of protamine and albumin seems to significantly improve the stability of nanoparticles in solutions with higher ionic strength, improving their internalization by cells [121].

Finally, we should mention the interest in functionalizing nanosystems based on protamine. Specifically, coating protamine-oligonucleotide nanoparticles with apoliporotein A1 increased protamine-AS-ON particle uptake and transcytosis in an *in-vitro* model of the blood-brain barrier [122]. Protamine modified with pullulan was also proposed for pDNA delivery, in order to protect the DNA from nucleases and prevent interactions with plasma components. Transfection experiments revealed the ability of the developed systems to provide high levels of gene expression [123]. Modification of protamine with stearyl groups also resulted in higher transfection efficiency, presumably due to the increased entry into cells facilitated by the corresponding hydrophobic interactions and subsequent easier unpacking of DNA from the internalized carriers [124].

8.3.1.4 Other Proteins

Some other endogenous polymers have not yet consolidated their use as biomaterials for nanoparticulate-based gene delivery, but they offer interesting possibilities that may be exploited in upcoming years.

Elastin-like polypeptides are polymers that consist of alternating hydrophobic blocks and crosslinking domains. Such polymers can be produced recombinantly and are composed of the repeating amino acid sequence (Val-Pro-Gly-Xaa-Gly)*m*, where Xaa is a hydrophobic domain that facilitates both self-aggregation and elastomeric functions. Thus, they undergo an inverse phase transition, which can be used to promote temperature-dependent self-assembly [125].

β-Casein is the most abundant milk protein component. Due to its amphiphilic nature, β-casein can establish intermolecular interactions and easily self-assemble into micellar structures. Around 15 to 60 β-casein molecules are required to form micelles with an average radius of 7–14 nm. However, changes in environmental conditions such as temperature, pH, ionic strength, water activity and hydrostatic pressure lead to changes in the size distribution of casein micelles due to the absence of a rigid three-dimensional tertiary structure [89].

8.3.2 Carbohydrates

Another important group of endogenous polymers are carbohydrates. Endogenous carbohydrates of interest for gene therapy are usually negatively charged due to the presence of either carboxylic acid or sulfate groups. Due to this fact, they can contribute two major functions to nanoparticle formulations. The first is to reduce toxicity—usually associated with positively charged compounds used for transfection—by charge neutralization. The second is the ability to interact with specific receptors related to cell adhesion or internalization of macromolecules. Among the carbohydrates used, Figure 8.5 shows the structures of polysialic acid and the glycosaminoglycans chondroitin sulfate, heparin and hyaluronic acid. They are the main endogenous carbohydrates used as biomaterials in the preparation of nanoparticulate systems for gene delivery.

8.3.2.1 Chondroitin Sulfate

Chondroitin sulfate is a widely distributed linear glycosaminoglycan that plays an important role in connective tissue and metabolism. It is composed of alternating units of N-acetyl-D-galactosamine and D-glucuronic acid with different degrees and patterns of sulfonation. It has been proposed as a biomimetic agent for rational gene delivery since it can improve the behavior of nanoparticle gene carriers by decreasing their cytotoxicity, preventing non-specific interactions with serum proteins and improving their transfection efficiency. In this respect, and as a negative polymer, chondroitin has been used successfully to decrease the intrinsic toxicity associated with positively charged

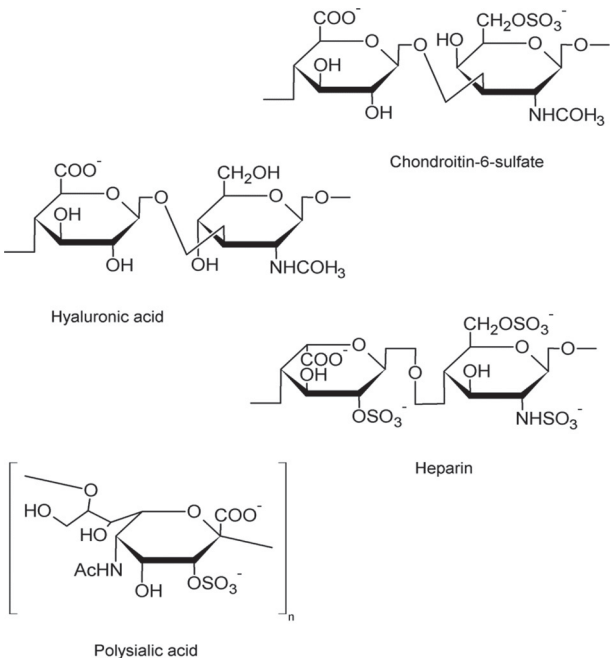

Figure 8.5 Structures of the main endogenous carbohydrates used as biomaterials in the preparation of nanoparticulate systems for gene delivery.

components used in non-viral gene therapy. Even the addition of small amounts of chondroitin sulfate was reported able to reduce the cytotoxicity to human corneal cells of cationized gelatin nanoparticles associated with pDNA 3-fold [115]. In the case of polyethyleneimine-pDNA complexes, chondroitin sulfate was able to reduce the cytotoxicity to B16-F10 mouse melanoma cells 5-fold [126,127]. In addition, as previously mentioned, nanostructures that present chondroitin sulfate in their composition can be specifically taken up into cells through their interaction with the internalization receptors CD44 and HARE receptors [128,129].

When used as a coating agent for nanoparticulate functionalization, the molecular weight and degree of sulfonation of chondroitin sulfate may be critical for the mean size of the final nanostructure. Thus, it has been reported that the lower the molecular weight, the greater the size of the final formulation (up to 1μm), although molecular weights higher than 22 KDa do not seem to affect the size. On the contrary, an increase in the degree of sulfonation can strengthen the interactions between smaller chains, contributing to diminished particle size. On the other hand, neither the size of chondroitin sulfate chains nor the extent of their sulfonation seems to influence the zeta potential of the developed nanoparticles [130,131].

Recently, chondroitin sulfate-cationized gelatin hybrid nanoparticles have been proposed for ocular gene therapy intended for topical administration. A plasmid codifying the mucin MUC5AC—essential for tear homeostasis but found at a decreased level in dry eye—was successfully transfected *in vitro* into cornea and conjunctiva cells [113,114]. Furthermore, these chondroitin sulfate-based nanoparticles were able to specifically transfect conjunctiva of rabbits and mice, as shown in Figure 8.6. *In vitro* cytoxicity and *in vivo* tolerance studies also showed that the system was innocuous under the conditions tested.

8.3.2.2 Hyaluronic Acid

Hyaluronic acid, or hyaluronan, is a non-sulfated non-epimerized, linear, endogenous glycosaminoglycan existing *in vivo* as a polyanion of hyaluronic acid and composed of

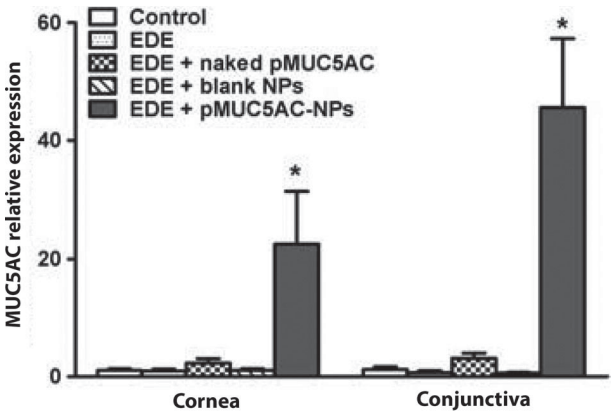

Figure 8.6 Expression of MUC5AC in experimental dry eye (EDE) mouse cornea and conjunctiva treated with pMUC5AC-NPs (EDE+MUC5AC-NPs). Controls include non-treated healthy mice (control), control EDE mice (EDE), EDE mice treated with naked plasmid (EDE naked pMUC5AC) and with blank NPs (EDE-blank NPs). EDE mice treated with pMUC5AC-NPs showed significant MUC5AC expression in cornea and conjunctiva (*$P<0.05$). Adapted with permission from [113].

repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine. It is a relatively safe compound, being widely used in cosmetic formulations and in regenerative medicine due to its low toxicity [132], as well as in nanotechnology to reduce the toxicity associated with other nanoparticle components. However, the most attractive property of hyaluronic acid as a constituent of a gene delivery system is its ability to interact strongly with various receptors, for example, the HARE and CD44 receptors, as previously stated [35,40,84–87].

With respect to hybrid hyaluronic acid nanoparticles, the high efficiency seen in the transfection process was due to the interaction of hyaluronic acid with CD44 receptors, as illustrated in Figure 8.7. The transfection effect seemed to last at least 10 days, reaching its maximum level on the fourth day after transfection of human corneal cells (HCE) and human conjunctiva cells (IOBA-NHC) [13,133]. This was evidenced by blocking (using an antibody or excess HA) these receptors, which led to a dramatic decrease in the transfection efficiency.

As observed with other polymers, the activity of hyaluronic acid seems to be influenced by its molecular weight. Hyaluronic acid oligomers are more effective than their counterparts with higher molecular weight, polymeric chains with molecular weights less than 10 kDa being more effective in the transfection of HCE and IOBA-NHC cells [13,133,134].

8.3.2.3 Other Carbohydrates

Heparin is a highly sulfonated and negatively charged glycosaminoglycan, best known for its use as anticoagulant. It is composed of several disaccharide units, especially 2-O and 6-O-sulfated iduronic acid. Hybrid nanoparticles of heparin and polyethyleneimine have been used to efficiently transfect plasmids encoding ms-T34A or vesicular stomatitis virus matrix protein, leading to reduced C-26 (colon cancer) cell proliferation by induction of apoptosis *in vitro*. Intratumoral injection of these nanoparticles

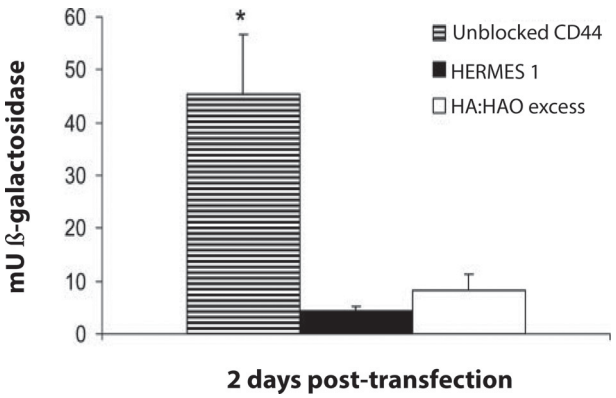

Figure 8.7 Transfection efficiency of pDNA-loaded NPs (HA:CSO 1:2) incubated with HCE cells. The CD44 receptor was blocked with 1 μg of the monoclonal antibody Hermes-1 or with an excess of a HA/HAO mixture (50-fold with respect to the amount of HA in the nanostructures). The expressed β-galactosidase was quantified by the ONPG reaction (mean ±SD, $n = 3$). (* Significant difference, $P < 0.05$). Adapted with permission from [133].

significantly inhibited the growth of subcutaneous C-26 carcinoma *in vivo* by inducing apoptosis and inhibiting angiogenesis [135], while intraperitoneal injection inhibited abdominal metastases of C-26 colon carcinoma through apoptosis induction and prolonged the survival of treated mice [136]. The function of heparin seems to be only to decrease the inherent toxicity of polyethyleneimine rather than stimulate transfection or internalization as other carbohydrates that were previously mentioned [137]. The most important disadvantage of using heparin is, however, its strong biological effect as an anticoagulant.

Polysialic acids are linear polymers of *N*-acetylneuraminic acid (sialic acids) units linked via α-(2→8) glycosidic linkages. They are abundantly present on the surface of cells [42]. Due to its high hydrophilicity, polysialic acid has been proposed as one natural substitute for polyethyleneglycol (PEG) in the development of second-generation stealth systems (drug delivery systems that present a reduced clearance) [138]. On the basis of these properties, polysialic acid has also been described as able to improve the half-life of hormones, growth factors and enzymes that, as nucleic acids, are very delicate molecules. With respect to PEG it should be emphasized that although products of PEG biodegradation are known to stimulate the immune system, leading to undesired reactions, such effects have not been reported for polysialic acid [139].

8.4 Conclusion and Future Perspective

Despite extensive research in the field, the major problem in gene delivery is to provide suitable spacial-temporal nucleic acid delivery or, in other words, the right nucleic acid concentrations at the site of action for a sufficient period of time. As we can conclude from the data discussed in the present chapter, nanotechnological approaches based on endogenous polymers as biomaterials offer a great opportunity to circumvent most of the significant barriers that limit broad clinical application of gene therapy. This is the reason why a great number of biomaterials and modifications of the same have been recently proposed for the development of nanotechnology-based gene delivery systems. However, the selection of the right biomaterial depends on several factors, such as availability and affordability at pharmaceutical grade, the nature of the nucleic acid to be associated and delivered, the type of system and the target tissue, among others. Therefore, a rational design of nanoparticle components and their final physicochemical properties is required. In addition, the extensive and practical use of endogenous proteins will depend on advances in recombinant technology, to avoid safety issues, as well as reductions in cost to make their use competitive. In any case, what is probably a statement of the obvious is that nanoparticulate carriers based on endogenous polymers should be prepared using mild conditions. Taking those premises into account, the use of low molecular endogenous amines as crosslinking agents has enormous potential, while modification of the polymers, even though possible, should be the last alternative to avoid complications in terms of safety, approval and large scale production. In such a scenario we can envisage non-modified anionic polymers, such as chondroitin sulfate and hyaluronic acid, and endogenous polyamines as future leading players. In addition, polysialic acid, which has only been fleetingly evaluated, is very promising or, at least, its promise remains uncertain but provocative. Specifically, it is our prediction

that in the years to come several next generations of naturally-based nanotemplates with specific architectures that mimic the cell surface biochemical demands of target tissues will be developed for gene delivery, which can be denoted as "back to nature" gene delivery strategies.

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9

Molecularly Imprinted Polymers as Biomimetic Molecules: Synthesis and Their Pharmaceutical Applications

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Abstract
After presenting a brief account of the history of the field, a general overview of the issues pertaining to the synthesis of the molecularly imprinted polymers and their inherent properties, the ingredients required for the synthesis of the molecules, the imprinting modes used in the preparation, and the effects of each on the properties of MIPs are provided. Next, considerations and mechanisms of the polymerization reactions applied to the preparation of MIPs and the physical forms in which the imprinted polymers are commonly prepared and used are covered. Finally, a general overview of the recent research on the applications of molecularly imprinted polymers in the delivery of pharmaceuticals, as well as the separation of drugs from different matrices and devices for the selective sensing of pharmaceutical species is provided. The text tends to be as comprehensive as possible and also includes some detail on the results of the cited works.

Keywords: Molecularly imprinted polymer, biomimetic molecules, chemical sensor, drug delivery, pharmaceutical separation, imprinting mode, polymerization, smart polymers

9.1 Introduction

The work on molecular imprinting started as a result of efforts made to induce selective affinity towards dye molecules in silica gel, with the aim of creating the ability of selective binding between the species [1,2]. The prototypes of such molecules were previously found among enzymes and antibiotics [3,4]. The process of imprinting molecules includes the addition of desired species to the solution of the monomers, so as to act as templates around which the monomers can arrange to form a target-specific cavity, based on the chemical affinities among the different species present during the polymerization. Naturally, once the polymerization reaction is over, the target species are removed. As a result there exists a cavity, which is not only a size-wise fit to the target species due to its steric geometry, but also offers target-specific interaction between the functional groups

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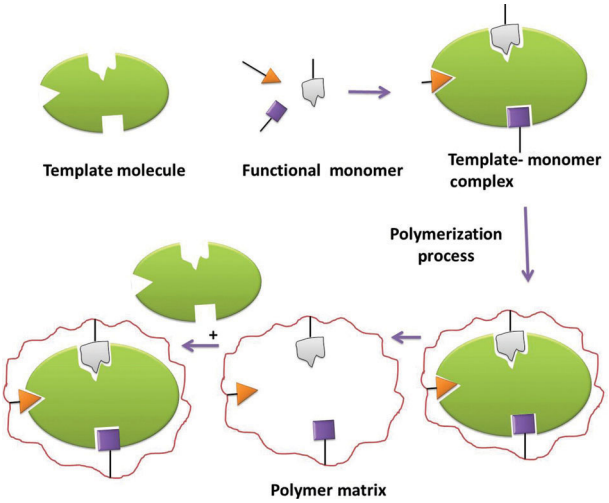

Figure 9.1 Schematic illustration of a molecular imprinting process.

of the monomers and the target species, the latter resulting from the alignment of the functional groups in favor of optimum interactions between the cavity and the target species in the ploymerization stage. Removals of the template are achieved through a range of techniques from washing with a solvent to a combination of chemical treatments coupled with washing, so as to totally or maximally remove the template from the imprinted molecules (Figure 9.1).

9.2 Preparation of Molecularly Imprinted Polymers (MIPs)

9.2.1 Reaction Components

Apart from the solvent, the components required for the imprinting process include the template and the chemicals participating in the polymerization reaction, namely the monomer(s), crosslinking agent and polymerization initiator. As in any other chemical reaction, these components play a crucial role in the compatibility of the reaction and its products with what is expected of the products.

9.2.1.1 Template and Monomer

As is obvious from its name, the species used as the template can have the most marked effect on the product of the imprinting procedure, since the properties of the cavity formed in the 3D structure of the MIPs is inherited from both the physical dimensions and outline of the template, as well as its chemical properties, which give rise to the complementary alignment of the functional groups in the monomers to best fit the template. Hence the most basic step would be choosing the template. Of course, since the removal of the template from the product is never complete, in cases where

the product is to be used for trace analysis of a target species the template species are sometimes chosen from the groups of compounds that have analogous properties with the target species. Such templates are also referred to as template mimics, surrogate or dummy templates [5]. This route helps avoid the risk of contaminating the sample solutions due to the probable release of the residual template species, but can also be adopted when the target species per se cannot be used as the template for some reason. The chemical properties of the template and the effects it can have on the reaction and the fact that it should not have the potential to chemically bind to the resulting MIP, or be chemically damaged or altered under the reaction conditions, are also of vital significance in the choice of templates.

The nature of the monomer, on the other hand, can naturally have a great influence on the properties of the MIPs, due to the variety and density of the functional groups present in its makeup. It is also likely that the monomer might form transitional complexes with the template [6,7] prior to the initiation of the polymerization reaction, which can have adverse or positive effects on the reaction and should henceforth be of concern.

An approach taken in the preparation of the MIPs is the so-called "generic approach," in which monomers are grafted with functional groups of a complementary nature with the target compounds (e.g., for acidic or basic templates, monomers with basic and acidic functional groups are chosen respectively) [5]. However, the choice of a proper monomer can be a formidable task. Conventionally this is done either based on the information in the literature or through trial and error, both of which might be rather laborious and ambiguous. So, three major approaches have been introduced to help with a more systematic and wiser choice of the monomers. These include the combinatorial [8,9], chemometric [10,11] and molecular modeling approaches [12–14].

9.2.1.2 Matrix-Forming or Crosslinking Agent

As pointed out earlier, once the monomers have effectually fashioned a cavity matching the properties of the template the reaction of the monomers starts a so-called matrix-forming [15] crosslinking agent. This leads to the stabilization of the structure of the MIPs through the formation of 3D bounds between the monomers and this reagent around the template.

Like any other chemical reagent, the nature and amount of this element can significantly affect the reaction and hence the properties of the product [10,16]. In short, increasing the relative amount of the matrix-forming or crosslinking agent decreases the elasticity and swelling properties of the MIPs due to the increased number of crosslinks in the three-dimensional structure of the polymer. These effects can be amplified by the inherent flexibility of the monomers, i.e., larger, more flexible matrix-forming agents and monomers lead to the development of more elastic and hence less template-selective MIPs, while the reverse is true for crosslinkers with lower molecular weights and chain lengths [5,17,18].

9.2.1.3 Porogenic Solvent

Due to its role as the medium, where the reaction takes place, the solvent is also very effective on the chemical, as well as the physical properties of the end product imprinted

polymers. The solvents commonly used, which are technically referred to as porogenic solvents, are favored due to the fact that they lead to the formation of pores in the structure of the polymers. Such porous structure of the product facilitates the mass transfer phenomenon throughout the polymers during their application, which increases the access of the target species to the cavities present in the MIPs.

The properties of the solvent can also impact the interactions among the monomers and the template complexes formed in the pre-polymerization stage in the noncovalent imprinting mode, which will be described later in the text. So the choice of the imprinting approach is highly dependent on the nature and physical properties of the solvents [17,19,20].

9.2.1.3.1 The Memory Effect

Based on experimental observations, if the interactions between the functional monomer and the templates are majorly hydrogen bounds, the solvent where the MIP and the target species are rejoined should be identical with the solvent used in the polymerization stage in order to have the best target recognition by the MIPs [21,22].

9.2.1.3.2 The Hydrophobic Effect

When assessing molecules imprinted with different triazine herbicides of diverse basic and hydrophobic properties, in the case of media with low aqueous content the basic character, of the template was found to lead to affinity and selectivity, while in the case of media with more aqueous content the role of the hydrophobic character of the template was more dominant. What was concluded was that based on the extent of hydrophobicity of the template, it induces specific or nonspecific effects on the recognition of the target by the MIP [23]. There have also been reports on the mere hydrophobic nature of the recognition in aqueous media [24–26].

9.2.1.3.3 Ion Exchange Phenomena

There have been reports on the fact that the binding of templates with protolytic functional groups to the sites in MIPs is of an ion-exchange nature. This phenomena was observed during the process of optimizing the mobile phase for the resolution of D,L-PheNHPh on an L-imprinted polymer as the stationary phase [27]. By moving from an organic mobile phase to a mixture of aqueous and organic mobile phases the efficiency of the chromatographic separation was increased. A second observation was that the sample load capacity was lower; probably because of the fact that the polymer shrinks, leading to closing of the micropores, which have slowly exchanging binding sites. Another effect reported was that lower pH values improved the selectivity, but this effect dropped by approaching pH values near the pK_a of the imprinted polymer [26].

9.2.1.4 Initiators and Inhibitors

Most of the MIPs are fashioned through the free-radical polymerization (FRP) procedures that are initiated upon the decomposition of a radical producing compound, referred to as the initiator, by means of thermal or photochemical stimuli. Such reactions are commonly conducted under mild temperature and pressure conditions. The FRP is appropriate for a variety of functional monomers and templates [28]. According to the general pattern of free-radical reactions, after the initiation phase, the number of the free radicals increases, leading to the propagation stage where the main portion of

the polymerization reaction takes place. The concentration of the free radicals in this stage can affect the polymer chain lengths through influencing the number of competing polymer chain. This means that more radicals decrease the chances of having fewer longer polymer chains, while in the case in which the concentration of the radicals is lower, the opposite can be true.

The most common initiating agents used in the preparation of MIPs are azo compounds like the frequently used N-N'-bis isobutyronitrile (AIBN) and 2,2'-azobis-(2,4-dimethylvaleronitrile); peroxo compounds like hydroperoxides, dialkyl peroxides, diacylperoxides and peresters oxidation-reduction systems like organic/inorganic peroxide redox couples and low valency metallic or nonmetallic compounds; as well as photolabile compounds with the tendency to absorb light, either directly or as a result of the presence of a photosensitizer [5].

The propagation stage of the FRP can be controlled either through controlling the intensity of the light received by the initiating agents or through the use of inhibitig species like oxygen, quinines like (R)-(6-Methoxyquinolin-4-yl)((2S,4S,8R)-8-vinylquinuclidin-2-yl)methanol, nitro compounds, and salts like $CuCl_2$ or $FeBr_3$ [5]. As stated previously, controlling the extent of free radical formation in the propagation period of the FRP is a means of adjusting the molecular mass and length of the end product through tampering with the speed of the reaction.

9.2.2 Imprinting Modes

The interactions of the species used as the template for the formation of the cavities in the structure of the imprinted molecules are important and defining parts in the process of forming MIPs. These interactions may even need to be tampered with and altered during the course of reaction, since although rather strong monomer-target interactions are desired until the very end of the polymerization stage, the same can be troublesome at the template removal stage, negatively affecting the characteristics of the produced polymer [29]. In light of these considerations, the combination of the template and the monomers is performed through different modes, to help with controlling the extent and strength of the template-monomer interactions.

9.2.2.1 Covalent Imprinting

As one of the modes of template-monomer interaction, covalent imprinting (CI), or otherwise referred to as the pre-organized approach [6], refers to procedures in which one or some monomeric species are joined through reversible covalent bounds to create the so-called template-monomers before the main polymerization or copolymerization process starts, through adding the required reagents (the crosslinker) and inducing the reaction conditions.

The different covalent interactions used in the CI of MIPs have been classified in many references [30–35]. In the case of the MIP produced through this strategy, the removal of the template commonly requires chemical treatment of the produced MIP. Covalent imprinting has been further categorized into different branches [29].

Condensation of readily reversible covalent bonds, which is the classical method of covalent imprinting, is one of these methods, where the template monomers are

prepared using condensation reactions. One example is the application of boronate ester for imprinting templates such as sugars like mannose derivatives, galactose and fructose [34,36], sialic and glyceric acids [37,38], castasterone, l-DOPA and nucleotides [33,39,40], ketal/acetal [41,42]; and Schiff's base formation, used for the imprinting of amino acids [43,44]. In the case of these reactions, the template can be separated from the polymer under mild hydrolysis with aqueous solutions, but the structural requirements of the method makes it applicable to a limited number of templates [21].

A second major category of covalent imprinting, namely imprinting with strong covalent bonds [45–49], includes the application of strong covalent bonds (e.g., ester and amide bonds) for assembling template and monomer species. Next, the templates are removed through a chemical reaction with an appropriate chemical agent or hydrolysis. This approach has somehow led to the formation of another imprinting mode, namely the semi-covalent mode.

Some of the disadvantages of the covalent imprinting mode are the fact that it is applicable for a narrow group of monomers and templates like alcohols, amines, aldehydes, ketones or carboxylic acids [50], and also that the separation of the template from the MIP involves rather harsh procedures. This is also indicative that the rebinding of the MIP with the target species will be slow since this necessitates the reformation of covalent bonds [50,51].

9.2.2.2 Semi-Covalent Imprinting Mode

The semi-covalent imprinting mode is comprised of covalently binding the template to a monomer, while the rebinding of the template to the MIP happens noncovalently. This is accomplished through the application of an intermediate group that is present between the template and monomer during the pre-polymerization stage. The nature of the binding between this intermediate group and the template differs from that of the monomer and the intermediate group. Hence, once the polymerization is achieved, the template is removed through breaking its bound with the middle group, which is now at the imprinted site, and hence the future interactions of the template (target) will be with this intermediate group. So, although the interaction between the template and the monomers is rather resilient through the pre-polymerization phase, thanks to the strong bond formed between the intermediate group and the monomer, the template will interact with the MIP through a more reversible bond it forms with the intermediate each time [52–55].

An example of this is the use of methacrylate esterified templates, which can indirectly co-polymerize with the monomer through the methacrylate chain. After the completion of the polymerization reaction, the template is removed through the hydrolysis of its rather reversible ester bound with the metacrylate group, leaving the ester as part of the cavity. Therefore, during the future rebindings of the cavity and the template, the unesterified template and the polymer can merely interact through the hydroxyl groups of the template with the residual (meth)acrylic acid in the cavity [56].

This approach suffers inadequacies like the fact that the hydrolysis reaction is by no means simple and the interactions of the free template with the intermediate group in the template sterification during the pre-reaction stage may be sterrically different from the hydrogen bonding that is possible in the rebinding stage between the

template and the residual intermediate group in the cavity [57]. It is even likely that the hydrolysis of the template form the intermediate in the MIP structure is not as easy as it was in their free form, which dictates harsher treatments for their separation [58].

An approach taken to overcome these deficiencies is the use of the so-called sacrificial spacers [29]. In this method the intermediate species used during the pre-polymerization of the MIP to link the monomers and the template is chosen so that it is eliminated—or in other words sacrificed—during the removal of the template. This way not only is the template attached to the monomer, the chances of steric influences of the residual moeities during the future rebindings with the target species are also avoided. Instances of the common sacrificial intermediate species are spacers with carbonyl groups [59–70] or the less frequent instances of salicylate (2-hydroxybenzoate) [60,71], dimethyl silyl group of silyl ethers [72] and silyl esters [73].

9.2.2.3 Noncovalent and Nonpolar Imprinting Modes

This method, also referred to as the self-assembly approach [31], is based on noncovalent interactions between the template and the monomers and has proven to be very effective [74]. The first reports on the approach date back to the time when silica matrices were used for the imprinting process, however the first well-known examples might be the works of Mosbach *et al.* [75,76]. Based on this route, the template and monomers are arranged with respect to one another due to the electrostatic forces, dipole-dipole, ion-pair, London forces and/or hydrogen bonds in the pre-polymerization stage, and evidently similar interactions will be dominant during the future interactions between the target and MIP in the rebinding stage. The arrangements formed during the pre-polymerization phase are rather unstable due to the nature of the forces triggering them, and can undergo severe changes owing to the disturbances that their formation equilibria might face throughout the synthesis process. Therefore measures need to be taken to stabilize these arrangements [29].

A clear advantage of this approach is that it does not require cumbersome chemical modifications, and the subsequent disadvantages with respect to the complications relating to the intermediate species (spacers); as well as the ease of removing the template after the preparation of the polymers. Examples of using non-hydrogen bond hydrophobic interactions to imprint polymers have proven this approach to be very effective [25,77]. The model has been used in preparing imprinted polymer stationary phase ortho or para xylene with very good and stable recognition ability even at elevated temperatures [78]. The work of Hosoya *et al.* [79], in which the molecular imprinting toward anthracene used uniformly sized ethylene dimethacrylate, showed that although the polymerization reactions were expected to have considerably more imprinting effects at 0°C, such effects were higher at 70°C.

Polycyclic aromatic hydrocarbons were also used for imprinting polyurethanes based on Van der Waals interactions, and the resulting MIPs were used for the recognition of the templates [80]. Cholesterol has also been used as the template in the polymerizaton of β-cyclodextrin with diisocyanates in dimethyl sulfoxide [81]. In another work, the vinyl monomer of 6-O-α-D-glucosyl-β-cyclodextrin was imprinted with dipeptides [82] or cyclodextrin [83]. Other examples include imprinting acryloylamylose with bisphenol A and alkylphenols [84].

Noncovalent modes also use hydrophobic interactions for imprinting, and consequently, recognition of nonpolar target species in polar solvents [29]. The examples include the works on cyclodextrins (CD)-MIP [85,86], oligopeptides [87], peptides [88], amino acids [89] and steroids [90].

9.2.2.4 Metal-Mediated and Metal-Imprinting Modes

There have also been reports on the application of metal ions in the imprinting procedure. Metal ions can act either as the template (metal imprinting), like in the case of imprinted polymers designed for the construction of ion selective electrodes [61,70,91,92], or have a role in the interactions between the template and the monomers (metal-mediated imprinting) [93,94]. The latter mode is less common because preparing and isolating well-defined tertiary or higher metal complexes is rather cumbersome [29].

9.2.2.5 Imprinting Crystals

Crystals of calcium salts have also been used for imprinting polymers of carboxylic acid functional monomers. Instances include imprinting artificial polymers with calcite to act as a template for the growth of specific crystal phases [95]. It has been reported that the calcite-imprinted polymers induced the formation of calcite nuclei even when the reaction conditions favored the growth of its other polymorph, namely aragonite. Another example of such work is the nucleation of calcium oxalate crystals on an imprinted polymer surface [96] where 6-methacrylamidohexanoic acid/divinylbenzene copolymers imprinted with calcium oxalate monohydrate (COM) or dihydrate (COD) crystals were used to foster the nucleation of CaOx crystals. The results revealed that the polymers gave rise to the formation of crystals that were the polymorphs of the template crystals.

9.2.3 Polymerization

The most conventional method of polymerization used for the synthesis of MIPs is the free radical polymerization (FRP) in bulk or in solution, which was mentioned earlier. The method is applicable for a varied array of functional monomers and templates. As previously stated, the reaction starts through initiation with an initiator activated through thermal, photochemical or redox stimulators. Since thermal initiation requires temperatures above 40°C, which might randomly influence the structure of the template-monomer assemblies formed in the pre-polymerization stage, initiation of the polymerization using photoinitiators, which does not affect the kinetic energy of the reaction ingredients, is preferred [22,28,97,98]. However, it is noteworthy that the propagation stage of the reactions initiated through light, is slower than those with thermal initiation, affecting the conversion of the double bonds and consequently the size and swelling properties of the resulting MIP [15]. The choice and nature of the reaction components, including the template, functional monomer and the crosslinking agent, have been studied in detail in various works [5,10,28,29,99–105].

It is, however, significant to note that to control the structural properties of the product, the overall reactions have been modified to some extent [5]. Some of the specific methods devised this way, which can be regarded as autonomous independent classes, include the precipitation or dispersion polymerization involving the reaction under diluted conditions applied to the preparation of micro, sub-micro and even nanometer MIP beads [106–109]; the suspension polymerization where the reaction ingredients are stabilized in the form of suspending droplets using surfactants and the reactions take place in the droplets acting as microreactors leading to the formation of micrometer-range MIP beads [110–117]; and finally emulsion polymerization, [118] where the monomers are closed within micelles, but the stabilization is similar to the suspension polymerization, due to the presence of surfactants.

There are also MIP preparation methods where polymeric or organic supports are used for the preparation of MIP beads. These include the multistep swelling polymerization routes [119–122], the use of silica beads as scaffolds for the MIPs [123–128], and the application of the so-called core-shell particles [129–134].

9.2.4 Physical Forms of MIPs

Based on the methods of production and with an eye on the final applications of the MIPs, which can range from selective removal or detection of target species to chromatographic and sol phase separations and drug delivery mechanisms, MIPs are produced in different physical forms and structures, ranging from films and beads to nanostructures [135–140].

Monolithically structured MIPs that have been prepared through bulk polymerization procedures have been used for capillary electrophoresis [141–143] and solid-phase microextraction (SPME) [144], as well as other analytical techniques like the chiral analysis of propranolol [145] or in competitive radio-labeled MIAs [146] and liquid chromatography [17,20,31,69–72].

MIP beads prepared through different approaches have been used for chromatographic separations as well as solid phase extraction experiments [121–128]. MIPs prepared in the form of membranes through one of the methods of *in-situ* polymerization, phase inversion polymerization and composite blending are commonly used for sensing puposes [5] or for the selective separation of the target species. The major disadvantage of membrane configurations is the lack of physical stability of the almost 2D structure, which can damage the integrity of the cavities, affecting the sensitivity of MIPs. In some cases the stability of the membranes has been enhanced by adding linear polymers or oligomers to the mixture before polymerization so as to fortify the structure [134,147].

MIPs have also been synthesized in the form of submicro- and nanostructures through different procedures like electrical deposition, photolithography, mechanical microspotting, soft-litography and electrospinning [148–151] or other techniques [5], to provide the product with the unique properties of the fine structures originating from their higher surface areas. This makes them suitable apposite for application in the transfer of drugs or hosting catalytic reactions [148,152,153].

9.2.5 Removing the Template

As stated in Section 9.2.1.1, effective removal of the template after the polymerization reaction is of vital importance. This can be very hard, particularly if the template is bound with the polymer network, which mandates the application of rather harsh removal conditions [154].

Washing the polymer with a proper solvent is the most primitive and yet effective method of removing the templates. This can be done once or several times with an organic or inorganic solvent or a solution that can best separate the template from the MIP, together with heating or stirring [155,156].

In the case of organic solvents, Soxhlet extraction can be used to enhance the extraction capability of the solvent by pressurizing it. Other methods include using pressurized subcritical solvents, or the application of microwave-assisted extraction [5].

9.3 Applications of Imprinted Polymers

An application of MIPs is their use as the stationary phase in the chromatographic separations and determinations [157–163]. Rather specialized applications of MIP in chromatography include their use as chiral stationary phases for separation of enantiomers using HPLC [164–171] or capillary electrochromatography [172,173]. Further, the compounds have been used in solid phase extraction (SPE) [174–180], also referred to as MISPE, for a variety of samples [181–186]. The ability of MIPs to display a selective response towards the targets they have designed for, also makes them good candidates for being used as sensing materials in sensing devices of different types [187–213].

Apart from the catalytic applications of MIPs described in other works [118,152], the compounds have also been used for pharmaceutical delivery, as well as separation and detection purposes, which will be covered in the three independent sections below.

9.3.1 Imprinted Polymers in Drug Delivery

The fact that MIPs can be designed as greatly selective receptors for templates of desire has led to their applications for drug delivery purposes, where a drug which has already been used as the template in the synthesis stage is later released into the target tissue by means of the same MIP. The substantial capacity of MIPs for the target species, and the fact that MIPs can release the desired species over long periods of time, makes them good candidate for application in this area. It is noteworthy that the application of MIPs in drug delivery systems has not matured into medical tests yet, due to the sensitivity of the issue. One of the major reasons behind this is the fact that most synthesis routes used for the preparation of MIPs do not have the capacity to yield pharmaeutical grade products, and the presence of residual organic solvents in MIPs can lead to cellular harm [28].

Another requirement for MIPs to be used for this purpose, is that they should not only be resilient against the biological reactions they will be prone to in the body, they should also have proper structual characteristics, as well as drug release kinetics. Table 9.1 provides an overview of the research in the area in recent years [214–242].

Table 9.1 Some of the recent reports on the application of MIP in drug delivery systems.

Drug Name	Mip Ingredients and Synthesis	Application in Drug Delivery	Reference
nicotine	MIP were synthesized by a free radical polymerisation method using nicotine as the template methacrylic acid as the monomer and ethylene glycol dimethacrylate as the cross-linker	for the controlled transdermal administration of nicotine	[214]
pantoprazole	MIP was prepared in chloroform as a solvent, 4-vinyl pyridine as a functional monomer, and ethylene glycole dimethacrylate (EGDMA) as a crosslinker monomer under UV irradiation.	for the controlled release of pantoprazole	[215]
gatifloxacin	Molecularly imprinted poly(hydroxyethyl methacrylate) microspheres were prepared via precipitation polymerization, using gatifloxacin (GFLX), hydroxyethyl methacrylate (HEMA), and ethylene glycol dimethacrylate (EGDMA) as template molecule, functional monomer and cross-linker	sustained release of gatifloxacin	[216]
betamethasone	magnetic molecularly imprinted nanoparticles (MMIPs) were synthesized using N,N-p-phenylene bismethacryl amide as a cross linker and super paramagnetic core-shell nanoparticle as a supporter by precipitation polymerization. Novel cross-linking agents were synthesized by the reaction of methacryloyl chloride with p-phenylenediamine. Then, the Fe_3O_4 nanoparticles were encapsulated with a SiO_2 shell and functionalized with -CH=CH_2 and MMIPs were further prepared by using methacrylic acid as a functional monomer, N,N-p-phenylene bismethacryl amide as a cross-linking agent and betamethasone as template.	for the controlled release of betamethasone	[217]
risperidone	high selective imprinted nanoparticle polymers were prepared by a miniemulsion polymerization technique, using risperidone as the template, MAA as the functional monomers, and TRIM as the cross-linker in acetonitrile as solvent.	Controlled release of risperidone from nanoparticles	[218]

(*Continued*)

Table 9.1 Cont.

Drug Name	Mip Ingredients and Synthesis	Application in Drug Delivery	Reference
doxorubicin	a layer-by-layer self-assembly method for doxorubicin (DOX) imprinting of biocompatible microcapsules, which consist of carboxymethyl cellulose-(chitosan/alginate) which are able to encapsulate and deliver DOX.	For delivery and sustain release of DOX and this method can be used for other water-soluble drugs	[219]
salicylic acid	One organic MIP and one inorganic MIP based on the sol-gel process were synthesized. The organic MIP was prepared by radical polymerization using the stoichiometric functional monomer, 1-(4-vinylphenyl)-3-(3,5-bis(trifluoromethyl) phenyl) urea, which can establish strong electrostatic interactions with the -COOH of salicylic acid. The sol-gel MIP was prepared with 3-(aminopropyl)triethoxysilane and trimethoxyphenylsilane, as functional monomers and tetraethyl orthosilicate as the crosslinker.	For release of salicylic acid in aqueous environments	[220]
diclofenac	Both conventional and electroresponsive imprinted polymers were synthesized with methacrylic acid as the functional monomer and ethylene glycol dimethacrylate as the crosslinker	for electroresponsive drug-delivery device	[221]
salidroside	pH-responsive nano-carrier MSNs-PAA, possessing mesoporous silica nanoparticles (MSNs) cores and poly(acrylic acid) (PAA) shell-layers, vinyl double bonds modified MSNs were synthesized by using cetyltrimethylammonium bromide (CTAB) as templates, tetraethyl orthosilicate (TEOS) as silicon source, and 3-(trimethoxylsilyl) propyl methacrylate (MPS) as surface modification functionalities	A pH-responsive nano-carrier for controlled delivery of drugs	[222]
Omeprazole	The optimized imprinted polymer was prepared in chloroform as a porogen. 4-vinylpyridine and ethylene glycol dimethacrylate were selected as a functional monomer and a crosslinker, respectively. (Because of the instability of OMP under polymerization conditions and the inability of the molecule to form effective interactions with monomers, pantoprazole (PANTO) was used as a dummy template for the imprinting process.)	For OMP controlled release	[223]

salicylate	imprinted electropolymerization of pyrrole, in aqueous solution containing pyrrole and salicylic acid was done.	the release kinetics of salicylate as a model drug from imprinted electropolymerization	[224]
tetracycline	A polyacrylate tetracycline (TC) selective microporous molecularly imprinted polymer was prepared in three different porogenic solvents (chloroform, acetonitrile, and methanol) via precipitation polymerization, using methacrylic acid monomer, ethylene glycol dimethacrylate crosslinker, and TC as template.	For controlled release of TC	[225]
dorzolamide	2-hydroxyethyl methacrylate (HEMA) as a backbone monomer, ethylene glycol dimethacrylate (EGDMA) as a cross-linker monomer, methacrylic acid (MAA) as a functional monomer and dorzolamide (DZD) as the template molecule	For preparation of a molecularly imprinted soft contact lens, as a new ocular drug delivery system for Dorzolamide (Using MAA co-monomer and applying a molecular imprinting technique increase loading capacity and releasing properties of hydrogels. The optimized imprinted hydrogel can control the release process of drug in aqueous media.)	[226]
epirubicin	Two monomers: methacrylic acid (MAA) and methacrylamide (MAM) were selected by computational simulation from the four monomers and used for synthesized.	due to its magnetic property, amphiphilicity, good biomimetic recognition of EPI, high adsorption capacity and controlled release, the epirubicin M-MIPs synthesized in this study are suitable to be applied to a magnetic targeted drug delivery system	[227]

(*Continued*)

Table 9.1 Cont.

Drug Name	Mip Ingredients and Synthesis	Application in Drug Delivery	Reference
propranolol HCl	MAA (methacrylic acid) as monomer and EGDMA (ethyleneglycol dimethacrylate) as crosslinker and solvent polymerization technique were used.	for drug release (complex of drug with beta-cyclodextrin were prepared for the higher drug release.)	[228]
thiamine hydrochloride	The MIP was synthesized by mixing thiamine hydrochloride (template), Itaconic acid (monomer) and Ethyleneglycoldimethacrylate (crosslinker) using azobisisobutyronitrile as initiator in acetonitrile containing 3-methacryloxypropyltrimethoxysilane– Montmorillonite and sodium acetate buffer.	In vitro release of drug in intestinal fluid with a controlled manner	[229]
naproxen	A conducting molecularly imprinted polymer (CMIP) film, based on polypyrrole, was electrosynthesized. The film was prepared by incorporation of a template anion (naproxen) during the electropolymerization of pyrrole into a platinum electrode using the cyclic voltammetry method.	The CMIP films, as the electrochemically controlled solid-phase sorbent, were applied for the selective cleanup and quantification of trace amounts of naproxen from physiological samples.	[230]
dipyridamole	Imprinted polymers were prepared from methacrylic acid (MAA) (functional monomer), ethylene glycol dimethacrylate (cross-linker), and dipyridamole (DIP) (as a drug template) using precipitation polymerization method.	The in vitro controlled releases of DIP	[231]
Carbamazepine	functional monomer, MAA, thick walled glass tube containing CBZ dissolved in porogen were used. The crosslinker, Trimethylolpropane trimethacrylate, and initiator, azobisisobutyronitrile, were also used.	20% of loaded CBZ was released from the imprinted nanospheres within the initial 6 h, while another 80% of CBZ was released in the following 9 days.	[232]

bromhexine	imprinted polymers were prepared from methacrylic acid (functional monomer), ethylene glycol dimethacrylate (cross-linker), and bromhexine (as a drug template) using bulk polymerization method.	For controlled release of bromhexine	[233]
citalopram	Imprinted polymers were prepared from methacrylic acid (MAA; functional monomer), ethylene glycol dimethacrylate (EGDMA; cross-linker), and citalopram (as a drug template) using bulk polymerization method.	For controlled release of citalopram	[234]
(S)-omeprazole	S-(–)-Omeprazole base, methacryloyl quinine or methacryloyl quinidine, Ethylene glycol dimethacrylate, 2,2′-Azobis (isobutyronitrile), and perfluoro(methylcyclohexane) were added to chloroform containing of emulsifier (PFPS).	a pH-responsive drug delivery system for enantioselective-controlled delivery of racemic drug (S)-omeprazole	[235]
L-methyldopa	The molecularly imprinted polymeric microspheres (MIPMs) for L-methyldopa (LMD) were synthesized by precipitation polymerization. The influences of synthetic conditions such as polymerization temperature, the amount of initiator (2,2′-Azobis(isobutyronitrile)) and the property of template on the morphologies and diameter sizes of MIPMs were investigated in details.	for controlled release of L-methyldopa	[236]
aspirin	Magnetic MIPs were synthesized by the co-polymerization of methacrylic acid (MAA) and trimethylolpropane trimethacrylate (TRIM) around aspirin (ASP) at the surface of double-bond-functionalized Fe_3O_4 nanoparticles in chloroform.	for controlled release of aspirin (release profiles and release rate of ASP from the ASP-loaded magnetic MIPs indicated that the magnetic MIPs also had potential applications in drug controlled release.)	[237]

(*Continued*)

Table 9.1 Cont.

Drug Name	Mip Ingredients and Synthesis	Application in Drug Delivery	Reference
alpha-tocopherol	Polymers were synthesized using methacrylic acid as functional monomer, ethylene glycol dimethacrylate as cross-linker and 2,2′-azoisobutyronitrile as an initiator	for a controlled/sustained drug release in gastrointestinal simulating fluids	[238]
5-fluorouracil	MIP were synthesized using methacrylic acid (MAA) as functional monomer and ethylene glycol dimethacrylate (EGDMA) as crosslinking agent and 2,2′-azoisobutyronitrile as an initiator	For an in vitro controlled/sustained drug release in gastrointestinal and plasma simulating fluids	[239]
caffeine	azobenzene-based functional monomer, 4-[(4-methacryloyloxy)phenylazo]benzoic acid (MPABA), using caffeine as a molecular template and Trimethylolpropane trimethacrylate (TRIM) was used as the crosslinker	For near-quantitative uptake of the released caffeine upon irradiation	[240]
timolol	Soft contact lenses (diameter 14 mm, center thickness 0.08 mm) consisted of NN-diethylacrylamide (DEAA; main component of the matrix), methacrylic acid (MAA; functional monomer) and ethylene glycol dimethacrylate (EGDMA; crosslinker) were prepared by the conventional methodology (non-imprinted) or by applying a molecular imprinting technique using timolol as the template (imprinted ones).	For in vivo usefulness of molecular imprinting technology in therapeutic soft contact lenses capable of prolonging the permanence of timolol in the precorneal area (These results indicate that imprinted soft contact lenses are promising drug devices able to provide greater and more sustained drug concentrations in tear fluid with lower doses than conventional eyedrops.)	[241]

sulfasalazine	Spherical molecularly imprinted polymers (SMIPs) have been prepared via a novel precipitation polymerization using sulfasalazine (prodrug used in the diseases of the colon) as template. Ethylene glycol dimethacrylate (EGDMA) as a crosslinker, methacrylic acid (MAA) as a monomer and 2,20-azoisobutyronitrile (AIBN) as an initiator	The sulfasalazine was incorporated into SMIPs and into a spherical non-imprinted polymer (control), and then the release rate of the bioactive agent at different pH values was evaluated.	[242]

Ruela and coworkers developed a transdermal MIP nicotine delivery system using a free-radical polymerization method using methacrylic acid monomers and ethylene glycol dimethacrylate crosslinker [214]. In another work, Mohajeri *et al.* reported the development of a pH-sensitive pantoprazole MIP and evaluated its pantoprazole binding and releasing properties in aqueous media. They tested different functional monomers during the MIP synthesis stage, including methacrylic acid, methacrylamide, hydroxyethyl methacrylate, and 4-vinyl pyridine, and also studied the reaction parameters, and found that 4-vinyl pyridine functional monomers lead to the best MIPs. They reported using chloroform as the solvent and glycole dimethacrylate as the crosslinking agent [215]. Another pH-responsive nano-sized drug carrier composed of mesoporous silica nanoparticles cores and poly(acrylic acid) coating, has been developed for the delivery of salidroside. In this study, mesoporous silica nanoparticles were synthesized using cetyltrimethylammonium bromide as the templates, and tetraethyl orthosilicate as the source for silicon. The surface of these organic cores was modified using 3-(trimethoxylsilyl) propyl methacrylate [222].

Lu *et al.* reported the preparation and characterization of molecularly imprinted poly(hydroxyethyl methacrylate) microspheres for sustained release of gatifloxacin. They reported the use of gatifloxacin, hydroxyethyl methacrylate, and ethylene glycol dimethacrylate as the template molecules [216].

Azodi-Deilami *et al.* prepared core-shell magnetic molecularly imprinted polymer nanoparticles (MMIPs) using N,N-p-phenylene bismethacryl amide crosslinking agent for the controlled release of betamethasone. They reported encapsulation of Fe_3O_4 nanoparticles with a SiO_2 shell and its functionalization with -CH=CH_2 and methacrylic acid as a functional monomer. In another report on Fe_3O_4-based magnetic MIPs, nanosized MIP beads with combined properties of molecular recognition and controlled release were prepared through the copolymerization of methacrylic acid and trimethylolpropane trimethacrylate in the presence of aspirin templates [217,237]. Another magnetic nanostructured MIP was introduced by Dramou *et al.* for the delivery of anticancer epirubicin, using two monomers of methacrylic acid and methacrylamide (MAM) based on computational estimations [227].

Asadi *et al.* described the synthesis of highly selective imprinted nanoparticle polymers for the controlled delivery of risperidone, using risperidone as the template and TRIM as the crosslinker in acetonitrile as solvent [218]. The preparation and application of hollow polysaccharide microcapsules coated with a MIP layer for the controlled release of water-soluble drugs were also reported by Wang *et al.* The researchers described a layer-by-layer self-assembly method for imprinting the microcapsules involving the use of carboxymethyl cellulose-(chitosan/alginate) with doxorubicin [219]. In another work, silica sol-gel molecularly imprinted polymers were developed for the delivery of salicylic acid. The researchers developed one organic MIP based on 1-(4-vinylphenyl)-3-(3,5-bis(trifluoromethyl) phenyl) urea and one inorganic MIP based on the sol-gel process. The MIP was prepared with 3-(aminopropyl)triethoxysilane and trimethoxyphenylsilane monomers and tetraethyl orthosilicate as the crosslinking agent. It was observed that the the organic MIPs had specific interactions with the target species in acetonitrile, while these interactions were weaker in the presence of water [220]. Puoci *et al.* developed MIP microspheres based on methacrylic acid as the functional monomer and ethylene glycol dimethacrylate as the crosslinker. The MIPs

were doped with carbon nanotubes to help with the so-called electroresponsive delivery of diclofenac. The electroresponsive delivery of the product was tested at a 20-V direct-current voltage applied through the releasing MIP media [221].

Tabassi *et al.* reported the preparation of an omeprazole (OMP)-imprinted polymer. However, due to the stability issues of the template under reaction conditions, a mimic template, namely pantoprazole (PANTO), was used in the imprinting stage in chloroform, further using 4-vinylpyridine as the monomer and ethylene glycol dimethacrylate as the crosslinker [223]. There is another report on an S-(–)-Omeprazole releasing system using methacryloyl quinine or methacryloyl quinidine, ethylene glycol dimethacrylate, 2,2′-Azobis(isobutyronitrile), and perfluoro(methylcyclohexane). The drug-release system was prepared using synthesized composite latex, and designed to comprise a so-called pH stimuli-responsive poly(hydroxyethyl methacrylate) and polycaprolactone-triol (PCL-T) blend, which was designed for the pH selective release of the target [235]. Imprinted polymers of pyrrole and salicylic acid prepared through electropolymerization have also been reported for the controlled delivery of salicylate [224].

In another work, polyacrylate tetracycline imprinted microporous polymers were prepared using methacrylic acid monomer and ethylene glycol dimethacrylate crosslinker. All polymers prepared in diverse solvents were reported to possess good releasing properties for the template [225]. Dorzolamide releasing MIPs were prepared using 2-hydroxyethyl methacrylate and methacrylic acid as the backbone and functional monomers respectively, and dorzolamide molecule was used as the template [226]. Barde *et al.* published an article on the development of an MIP for the sustained release of propranolol HCl using methacrylic acid as the functional monomer and ethyleneglycol dimethacrylate as the crosslinker [228]. In another report, silylated montmorillonite-based MIPs for the *in-vitro* release of thiamine hydrochloride were reported. Itaconic acid functional monomers were used in combination with ethyleneglycoldimethacrylate as the crosslinker [229]. Ameli *et al.* reported nanostructured conducting MIP films for the selective release and uptake of naproxen templates [230] based on polypyrrole. The sustained release of dipyridamole-based MIPs prepared using methacrylic acid functional monomers and ethylene glycol dimethacrylate has also been reported recently [231].

The effect of the solvent on the characteristics and release of carbamazepine MIP nanospheres and the syntheses and characterization of MIPs for the release of bromhexine has also been reported [232,233]. In the latter case, the MIPs were prepared using methacrylic acid monomers ethylene glycol dimethacrylate crosslinking agent.

A citalopram releasing MIP was reported by Abdouss *et al.* using the same functional monomer (i.e., methacrylic acid) and ethylene glycol dimethacrylate (EGDMA) as the crosslinker, together with the drug as the templat [234]. Ling *et al.* reported the synthesis and characterization of MIP microspheres with L-methyldopa templates through precipitation polymerization and used it for the controlled release of L-methyldopa. They evaluated the effect of the synthesis conditions, including the reaction temperature and the amount of initiator (2,2′-Azobis(isobutyronitrile)) [236].

Alpha-tocopherol and 5-fluorouracil imprinted polyers reported by Puoci *et al.* were prepared using common functional monomers, i.e., methacrylic acid and ethylene glycol dimethacrylate and 2,2'-azoisobutyronitrile as the crosslinking and initiator agents.

The authors reported the polymers to possess controlled/sustained release properties in biological fluids [238] [239]. Puoci *et al.* also reported spherical MIPs for the controlled delivery of sulfasalazine using the same functional monomers and crosslinkers but a different initiator, i.e., 2,20-azoisobutyronitrile (AIBN) [242].

Caffeine and timolol relesing MIPs were also reported by B. Gong *et al.* [240] and H. Hiratani *et al.* [241]. The former work was on the polymerization of 4-[(4-methacryloyloxy)phenylazo] benzoic acid as the functional monomer together with trimethylolpropane trimethacrylate (TRIM) as the crosslinker, in the presence of caffeine as the template [240]. In the latter, soft contact lenses consisting of N,N-diethylacrylamide, methacrylic acid as the functional monomer and ethylene glycol dimethacrylate as the crosslinker were prepared using timolol as the template. The lenses were tested in-vitro to assess the applicability of MIPs in the construction of therapeutic soft contact lenses capable of increasing the durability of timolol in the pre-corneal region [241].

9.3.2 Imprinted Polymers in Separation of Pharmaceuticals

Due to the selective and rather specific tendency of MIPs towards the templates, these structures have been used for the separation of different target species through a variety of methods [157–186]. In this section the studies performed on the separation of pharmaceutical species based on MIPs shall be covered [243–292] in some detail with respect to the polymer ingredients and the results obtained.

V. Thibert *et al.* developed an MIP for the extraction of cocaine and its metabolites, i.e. benzoylecgonine and ecgonine methyl ester from urine prior to LC-MS procedure. They used cocaine as the template during the polymerization of methacrylic acid monomers in combination with ethylene glycol dimethacrylate cross-linker. The polymerization reaction was performed in acetonitrile and azo-N,N/-bis-isobutyronitrile was used as the initiator. The LOQs for cocaine, benzolecgonine, and methyl ester, after separation and preconcetration through the MIPs, were evaluated to be 0.09 ng/mL, 0.4 ng/mL, and 1.1 ng/mL respectively [243].

In another work lacosamide based MIPs of methacrylic acid monomers were used for the solid-phase extraction of the template from rat plasma before LC analysis and the results revealed a recovery of over 98% for the SPE and the LOD and LR of the method were evaluated to be 0.03 μg mL^{-1} and 0.1–100 μg mL^{-1} [244]. B. B. Prasad *et al.* reported the MIP-based SPE of epinephrine and detection of the same through an MIP based sensing device in plasma cerebrospinal fluids. They reported the reaction of functionalized multiwalled carbon nanotubes (MWCNTs-COCl)] as a monomer with N-hydroxy phenylmaleimide and used glycoldimethylacrylate as the cross-linking agent. The LOD of the hyphenated method was reported to be 0.002 ng mL^{-1} [245]. In another study M. Moein and co-workers developed MIP cartridges to be used in combination with HPLC for facile analysis of human insulin in plasma and urine. They used insulin as the template, in the reaction between methacrylicacid monomer and ethylene eglycol dimethacrylate cross-linker. The reaction initiation was achived by 2,2/-azobisisobutyronitrile. The overal results showed LODs of 0.2 ng mL^{-1} in plasma and 0.03 ng mL^{-1} in urine with recovery factors over 87% [246].

E. Lendoiro *et al.* have recently reported the application of catechin as a mimic template, in the polymerization of 4-vinylpyridine monomers and ethylene glycol dimethacrylate cross-linker and used the resulting MIPs for the determination of delta(9)-tetrahydrocannabinol and 11-nor-delta(9)-tetrahydrocannabinol carboxylic acids in urine and saliva through LC-MS/MS. The method showed rather wide linearity ranges, i.e., 1–500 ng/mL and 2.5–500 ng/mL in saliva and urine respectively, with a reported accuracy of 98.2–107.0 % [247]. In another recent report Y. Lei *et al.* described a so-called MIP-coated stir-bar. The MIP used for coating the bar was based on the common methacrylic acid monomer, ethylene glycol dimethacrylate and trimethylolpropan trimethacrylate cross-linkers, polymerized in the presence of dopamine using 2,2′-azobis-(isobutyronitrile) initiation in methanol and water. The tool was used for the extraction and analysis of dopamine in human urine [248]. MIPs based on pioglitazone template, methacrylic acid functional monomer and ethylene glycol dimethacrylate as the cross-linker have also been used for the target-specific extraction of pioglitazone from cow plasma and its consecutive analysis through ESI ion mobility spectrometry. The method has been reported to have a linearity range of 0.10–20.00 μg/mL, and RSD of less than 6% and a recovery of 91% [249].

The selective extraction of catecholamines has been the subject of some studies [250,269]. Recently B. Claude *et al.* developed a method for the selective SPE of catecholamines and metanephrines from bovine serum using MIPs, and reported the recovery, RSD and linearity range of the method to be in the orderof 64–98%, less than 6% and 0.30–0.90 μM respectively [250]. In another study M. Bouri *et al.* reported the selective extraction of catecholamines from urine using a dopamine MIP coated magnetic molecularly imprinted polymer and capillary electrophoresis. They used methacrylic acid as the monomer, ethylene glycol dimethacrylate as the cross-linker and coated the MIP on Fe_3O_4 particles and used 2,2′- azobisisbutyronitrile as the initiator in methanol/water or acetonitrile/water solvents, and reported LOD of 0.04–0.06 μM, RSD of 0.7%–1.4% and a response range of 2.9%–5.5% [269].

Similarly gatifloxacin (GTFX) in human urine and water samples was separated using magnetic MIP coated particles. GTFX templates were used in the pre-polymerizationn of MIPs based on methacrylic acid functional monomers, and ethylene glycol dimethacrylate cross-linker, which were coated on Fe_3O_4 magnetic nano particles. The results showd a linearity range of 0.25–15 μg mL^{-1}, limit of detection of 0.075 μgmL^{-1}, and recovery and RSD values of 91% and 2.5- 3.3 respectively [260]. Another case of application of magnetic molecularly imprinted polymers (MMIPs) is the work of F.-F. Chen *et al.* who developed an MMIP for six synthetic phosphodiesterase type-5 (PDE-5) inhibitors, using sildenafil as the only template. The extracted species were analysed through an HPLC equipped with a diode array detector (DAD). The polymers prepared using sildenafil template were tested for the separation of sildenafil, vardenafil and their analogs in herbal and medicinal samples with LOD and recovery of 9.49 μg L^{-1} and 60.0–94.7%. The functional monomer was the commonly used methacrylic acid in combination with ethylene glycol dimethacrylate as the cross-linking agent, and 2,2′- azobisisbutyronitrile as the initiator in toluene [263].

Acetaminophen and aspirin specific MIPs have also been prepared based on methacrylic acid, 4-vinylpyridine and methyl methacrylate as the monomer and ethylene glycol dimethacrylate as the cross-linking aganet [251]. MIPs have further been used

for the solid-phase extraction and consecuent determination of artemisinin in artemisia annua L [252], or the extraction and analysis of acyclovir from serum with LOD of 1.8 ng/mL and recovery of 95.6% using the MIPs of allylcytosine monomer [253]. Methacrylic acid MIPs prepared in the presence of ractopamine, lamivudine, chlorpromazine and imiquimod templates have been reported for the separation orf the template species in biological matrices [254–257].

In another exeriment amidoamine as the monomer and ethylene glycol dimethacrylate as the cross-linker were used for the preparation of a naproxen imprinted polymer on magnetic multi-walled carbon nanotubes, which were used for the SPE of the same in human urine samples in the linear range of 4.0–40.0 ng mL^{-1} and with an LOD of 2.0 ng mL^{-1} [258]. MIPs have been further evaluated as the packing material for solid-phase extraction of glibenclamide in the quality control during the cleaning process in the production of dry drug forms. The polymers were prepared using 4-vinylpyridine monomers and ethylenedimethacrylate cross-linking agent, and the resuting MIPs showed recovery values of 87.1% [259].

S.M. Daryanavard *et al.* used a MIP prepared with a pentycaine template, for the microextraction and subsequent analysis of lidocaine, ropivacaine, mepivacaine and bupivacaine in plasma and urine. The functional monomer and crosslinking agents were methacrylic acid and ethylene glycol dimethacrylate, and the reaction was initiated by azobisisbutyronitrile in dry toluene. The results showed the linearity range and recovery of the method to be in the range of 5–2000 nmol/L, 60–80% [261]. Another example of using one analogue as the template for a series of analogues is the work of F. Chen *et al.* where sildenafil was used for the preparation of MIP particles with selectivity for six synthetic phosphodiesterase type-5 (PDE-5) inhibitors [263] or the work of D. Djozan *et al.* who developed methamphetamine as the template in the synthesis of methacrylic acid based MIPs used in an inside-needle adsorption trap for the adsoption of methamphetamine, amphetamine and ecstasy in human urine with LOD and recovery of 12 $ngmL^{-1}$, 81–93% in spiked human urine [267].

There is a report on the ELISA-development of an assay for vancomycin, where I. Chianella *et al.* proposed a simple method for coating microplate wells with MIP nanoparticles to develop ELISA type assays for vancomycin and used the resulting device for blood plasma samples. The polymers were prepared using N-isopropylacrylamide, N,N′- methylene-bis-acrylamide, N-tert-butylacrylamide and acrylic acid as monomers and tetramethylethylenediamine as the initator. The researchers used competitive binding with an HRP-vancomycin conjugate for the determination of the target compound and achieved a lineraity range of 0.001–70 nM with an accuracy level of 98% [262].

Other examples of MIPs prepared for the separation of pharmaceuticals using methacrylic acid as functional monomer and ethylene glycol dimethacrylate as the cross-linker include the MIPs with caffeine templates for the extraction of the same from human plasma and tea [264]; or MIPs with dipyridamole, methadone, citalopram, Propranolol, 3-methylflavone-8-carboxylic acid, dextromethorphan, benzodiazepines, methamphetamine, hydrochlorothiazide, tolazoline, tramadol, pseudoephedrine and promethazine templates for the extraction of the templates from urine, saliva and serum samples [266,270,271,274,275,277,279,280,284,287,288,290,291,292]. Further examples include MIPs of the same compositions prepared with dextromethorphan templates for

the extraction of the same from rat plasma and urine [276] or with naphazoline template used for the extrction of the template from nasal drop samples [283].

Another example of acrylamide based MIPs for pharmaceutical templates is the preparation of rhaponticin MIPs using acrylamide used as functional monomers in combination with styrene as a copolymer monomer, ethylene glycol dimethacrylate as a cross-linker and 2,2'- azobisisbutyronitrile as an initiator in dimethyl sulfoxide. The MIPs prepared this way were used for the extraction of rhaponticin in Chinese medicines with a recovery of 77.82–91.00% in spiked samples [268]. Kaempferol was also used as a template for the preparation of MIPs with acrylamide as the functional monomer, ethylene glycol dimethacrylate as the cross-linker and 2,2-azobisisbutyronitrile as the initiator in methanol/acetonitrile. The MIPs were used for the extraction of the template from traditional Chinese medicines within a linear range of 4.5-200 mg L^{-1}, LOD of 8.0 μg g^{-1} [272]. Another example of MIPs with the same ingredients are MIPs with podophyllotoxin templates [273]. Acrylamide, 4-vinylpiridine and acrylonitrile functional monomers, together with ethylene glycol dimethacrylate as the cross-linker and furosemide templates have also been used for the separation of the template from mixtures [289].

MIPs with other functional monomers have also been reported. Amiodarone MIPs based on 4-vinylpyridine monomer was used for the extraction of amiodarone from serum within a linear range of 0.1–10 mg L^{-1} with a precision of 3.2% for 1.0 mg L^{-1} [265]. Phenobarbital MIPs based on vinylpyridine as the functional monomer and and 2-O-meth-acryloyloxyethoxyl-(2,3,4,6-tetra-O-acetyl-beta-D-galactopyranosyl)-(1-4)-2,3,6-tri-O-acetyl-beta-D-glucopyranoside as a surface-modifying glycomonomer [278]; barbiturates MIPs based on ,6-bis-acrylamidopyridine as the monomer, divinylbenzene-80 as the cross-linker and 2,2-azobis-isobutyronitrile as the initiator [281]; erythromycin MIPs based on 3-aminopropyl-trimethoxysilane as the afunctional monomer, and tetraethylorthosilicate as a reticulating agent [282]; D- and L-thyroxine MIPs prepared using 1,3-diacroloylurea as the monomer, ethylene glycol dimethacrylate as the cross-linker and 2,2-azobis-isobutyronitrile as the initiator in dimethylsulphoxide [285]; and folic acid MIPs based on 2,4,6-trisacrylamido-1,3,5-triazine monomers, ethylene glycol dimethacrylate cross-linkers and 2,2-azobis-isobutyronitrile as the initiator in dimethylsulphoxide [286] are examples of other molecularly imprinted polymers prepared for the separation of pharmaceutical specieis. Other reports on MIPs for pharmaceutical separations have also been reported in the literature [293–340].

9.3.3 MIPs in Devices for Sensing Pharmaceutical Species

S.A. Piletsky, reoprted the first application of MIPs in sensors [341] and ever since then a great deal of hard work has been directed towards the development of sensing devices based on MIPs, due to the fact that the MIPs enjoy the feature of selectively binding to the target species for which they have been designed. This binding can lead to changes in some physical property like the mass which is the basis for acoustic sensors, refractive index exploited in surface plasmon resonance sensors, or impedance leading to the signals in electrochemical sensing [342], or the electrochemical potentials across membranes, that is typically used for the cosntruction of ion selective electrodes. The

following lines will cover a summary of the different MIP based sensors for pharmaceutical species [343–452].

Dopamine might be a pharmaceutical for which the highest number of MIP based sensors have been constructed. Voltammetry [361,413] and its derivative techniques like differential pulse voltammetry [347,395,418], differential pulse anodic and cathodic stripping voltammetries [353,387,415]; ion selective electrodes [372], as swell as piezoelectric microgravimetry [401] and thermogravimetry [427] are among the mechanisms used for the detection in dopamine selective MIP based sensors.

B. B. Prasad *et al.* developed a sensor based on an imprinted polymer sensor for the detection of ultratrace amounts of dopamine. One reason for the high sensiytivity of the method was the solid phase micro extraction step performed prior to the sensing stage which leads to a 0.018 ng mL^{-1} detection limit for this voltametry based senor [413].

Three MIP-based differential voltametric sensors for dopmine have been proposed including the works of Y. Zeng *et al.* [347], W. Song *et al.* [395], and J. Li *et al.* [418]. Zeng *et al.* reported the use of a composite of SiO_2-coated graphene oxide and MIPs based on, dopamine templates and methacrylic acid (the monomer); thyleneglycoldimethacrylate (the cross-linker) and 2,2-azobisisobutyronitrile (the initiator) in methanol and achieved linerity range and detection limits of 5.0×10^{-8} – 1.6×10^{-4} M and 3.0×10^{-8} M respectively [347] .In another work W. Song and co-workers prepared a dopamine sensor with the linerity range and detection limit of 5.0×10^{-7}–4.0×10^{-5} M and 0.13 μM respectively based on MIPs prepared through the electrocopolymerization of o-phenylenediamine and resorcinol in the presence of the target molecules as the templates, and using differential voltametric detection [395]. J. Li, *et al.* further reported a sensor for the same analyte based on a molecularly imprinted electropolymer of o-aminophenol with a linear response in the range of 2×10^{-8}–0.25×10^{-6} M and a detection limit of $1.98 \times 10^{(-9)}$ M in the presence of high concentrations of ascorbic acid as the interfering species [418].

Also differential pulse anodic and cathodic stripping voltammetry based MIP sensors for dopamine have been described in the literature [353,387,415]. After reporting an MIP dopamine sensor [415], B. B. Prasad, *et al.* reported the development of a sol-gel derived multiwalled carbon nanotubes ceramic electrode modified with molecularly imprinted polymer for dopamine as a new class of composite electrodes. They used the ceramic-multiwalled carbon nanotubes substrate for growing a nanometer range film using the so-called surface grafting approach. The surface of the electrode was initially modified with benzyl N,N-diethyldithiocarbamate before modification with the dopamine MIP [387]. The same reseacher and his team have recently coupled an imprinted stir bar sorptive extraction technique with with an MIP sensor of the same analyte. The MIP was prepared using p-nitrophenyl acrylate as the functional monomer, and ethylene glycol dimethacrylate as the cross-linker and a etection limit of 4.9 ng L^{-1} was achieved [353].

L. Gu *et al.* developed a boronic acid functionalized poly(aniline-co-anthranilic acid) MIP composite and used it for the determination of dopamine through impedance spectroscoy. They innovated the introduction of aminophenylboronic acids and vinyl groups onto the surface of poly(aniline-co-anthranilic acid) nanomaterials, before copolymerization of acrylamide and ethylene glycol dimethacrylate

the presence of dopamine. The sensor was tested in the presence of interfering species like norepinephrine, epinephrine, ascorbic and uric acids and showed good selectivity behavior with a detection limit of 3.33×10^{-9} M [361].

An ion selective MIP sensor for dopamine was introduced by M. Pesavento *et al.* [372]. This all-solid-state ion selective electrode was prepared through screen printing a graphite electrode which was modified with a multiwalled carbon nanotubes, with a dopamine selective MIP membrane based on methacrylic acid monomers and ethyleneglycole dimethacrylate cross-linker [372].

A. Pietrzyk *et al.* reported an imprinted poly[bis(2,2 '-bithienyl)methane] film for a piezoelectric microgravimetry of dopamine. The MIP film contained either a 3,4-dihydroxyphenyl or benzo-18-crown-6 substituent, for selective determination of dopamine and was electropolymerized on an underlayer of poly(bithiophene) on a Pt/quartz resonator. The detection limit of the method was reported to be 10 nM [401]. Kan *et al.*, on the other hand, developed a composite of multiwalled carbon nanotube (MWCNTs) and MIP with dopamine templates using the copolymerization reaction of methacrylic acid and trimethylolpropane trimethacrylate (copoly(MAA-co-TRIM)) on the vinyl functionalized MWCNT surface and used the composite for the thermogravimetric analysis of the template. The composite was found to be selective towards dopamine in comparison with epinephrine and the response was linear in the range of 5.0×10^{-7}–2.0×10^{-1} M [427].

Another pharmacetical of interest in the area of MIP-based sensors has been folic acid. B. B. Prasad *et al.* reported a series of folic acid MIP sensing systems using differential pulse cathodic stripping voltammtery in 2010 [286,398,399]. In one of their reports, they used an MIP fiber (monolith) for use in sensing devices, with the advantages of higher degrees of enrichment of folic acid, without surface fouling, cross-reactivity, and interferences from the species present in complex matrices. The MIPs were based on triaminotriazine functional monomers, and ethylene glycol dimethacrylate as a cross-linking agent and 2,2'-azobisisobutyronitrile was used as the initiator and the detection limit of the method was evaluated at 0.002 μg mL^{-1} [286]. In another work they developed an MIP-carbon consolidated composite fiber sensor to the same end. Apart from the template, 2,4,6-trisacrylamido-1,3,5-triazine monomers, and ethylene glycol dimethacrylate cross-linkers were used and the system led to detection limits in the range of 0.20 ng mL^{-1} [398]. Their next report described a folic acid sensor based on a hyperbranched MIP-immobilized sol-gel-modified pencil graphite electrode using 2,4,6-trisacrylamido-1,3,5-triazine as the functional monomer and ethylene glycol dimethacrylate as the cross-linking agent. They used a new apprach to avoid the deficiencies of the substructure imprinting of the larger molecule i.e. folic acid, which include the co-recognition for both folic acid and the analogues containing pteridine and glutamic acid substructures. They hence designed an MIP with the tendency of specifically binding folic acid through the so-called stoichiometric imprinting process, which gave birth to multiple binding sites within the cross-linked hyperbranched polymer. They created dendrimer-like chains using the 'initiator-fragment incorporation radical polymerization' technique with 2,4,6-trisacrylamido-1,3,5-triazine, which is a trifunctional monomer. The MIP was coated over a preanodized sol-gel coated pencil graphite electrode to yield a pencil graphite electrode with a detection limit in the range of 0.002 μg mL^{-1} [399].

Very recently a differential pulse voltammetry device has been developed through imprinting smart polymers. In this experiment, which was described by N. Karimian *et al.*, a temperature sensitive amine-terminated poly (N-isopropylacrylamide) block, and (N,N'-methylenebisacryl amide) cross-linker with o-phenylenediamine were electropolymerised on the surface of a gold electrode, using folic acid as the template. This led to a thermally switchable MIP sensor with selectivity towards the template [355].

In the case of detecting histamine, a fluorescent sensor was developed in 2002 by Tong and Dong *et al.* They reported the use of zinc (II)-protoporphyrin as a fluorescent functional monomer and methacrylic acid as a functional monomer for the recognition of histamine. The Zn binding sites in the structure of the functional monomer were reported to bind with the imidazolyl group of histamine through coordination. The decrease in the fluorescence intensity of the MIP upon its inteaction with histamine was used for the determination of histamine concentration from 0.1 to 1 mmol/l above which a saturation behavior was observed [449]. In 2009 Pietrzyk *et al.* developed a selective piezoelectric sensor for histamine using bis(bithiophene) derivatives to electrochemically prepare an MIP recognition film and a 10 MHz AT-cut "shear-thickness-mode bulk-acoustic-wave quartz crystal resonator" with Pt film electrodes as the transducer. To avoid the electro-oxidation of histamine and the possible contamination of the surface of the Pt electrode a protective poly(bithiophene) film was initially electrochemically deposited on the Pt/quartz resonator before coating it with the MIP of two functional monomers of bis(bithiophene) derivatives with 18-crown-6 and a dioxoborinane substituent respectively. The resulting combination showed linear response in the range of 10–100 mM with a detection limit of 5 nM [416]. One year later F. Horemans, *et al.* developed two impedance spectroscopic and microgravimetric MIP-based histamine-sensing "platforms" made of methacrylic acid monomers, ethylene glycol dimethacrylate cross-linker and 2,2′-azobisisobutyronitrile as an initiator in dimethylsulfoxide solvent. The electrochemical impedance spectroscopic platform was reported to be able to determine nanomolar range concentrations of the target species, while the microgravimetric one led to results in micromolar range [406].

Gonzalez *et al.* published three reports on the development and comparison of digoxin MIP-sensors in 2008 and 2009. The first report was on a flow-through MIP-fluorosensor for dioxin, based on MIPs of methacrylic acid monomers and ethylene glycol dimethacrylate crosslinker, using a photopolymerization reaction initiated with AIBN in acetonitrile. The results revealed a response linearity in a range of 1.0×10^{-3}–4.0×10^{-3} m gL^{-1} and a detection limit of 1.7×10^{-2} μ gL^{-1} [430]. Further, two automated MIP-based flow-through fluoroimmun and immunosensors were compared with an anti-digoxin polyclonal antibody for the determination of digoxin. It was stated that although the analyte was reproducibly determined by both devices, the MIP-based devices had a lower detection limit (1.20×10^{-3} mg L^{-1} versus 1.7×10^{-5} mg L^{-1}) [423]. Gonzalez *et al.* also reported an optical MIP-based sensor for digoxin with a detection limit of 3.17×10^{-5} mg L^{-1} in the same year [424].

Guo *et al.* reported a potentiometric tetracycline selective MIP electrode using tetracycline as template, methacrylic acid as the functional monomer, and ethylene glycol dimethacrylate as crosslinker during the polymerization of the MIP in a methanol-water

mixture, and reported a detection limit and response linearity range of 2.5×10^{-8} M and 6.0×10^{-8}–1.0×10^{-3} M respectively [407].

More recently, Wang *et al.* reported the electrochemical determination of tetracycline with the aid of "multiwall-carbon canotube-gold nanoparticle electrodes (MWNTs-GNPs)," coated with MIPs (tetracycline template, methacylic acid monomer, and ethylene glycol dimethacrylate crosslinker), and reported detection limits of 0.04 mg L^{-1} [383]. In the same year, Gai *et al.* reported a highly-sensitive MIP ion selective electrode (MIP-ISE) for the determination of tetracycline in the range of 2.0×10^{-8}–1.0×10^{-3} M with a detection limit of 1×10^{-8} M [392].

Morphin sensors based on MIPs have also been described [432,439,441]. Amperometric morphine sensors based on morphin imprinted poly(3,4-ethylenedioxythiophene), which catalyze morphine oxidation and lowers the oxidization potential on an indium tin oxide (ITO) electrode, is an example. The same MIP has been used in the form of immobilized molecular particles for the same purpose. In one report, rather uniform MIP microspheres were prepared through precipitation polymerization to produce more active surface area. Poly(3,4-ethylenedioxythiophene) was utilized to immobilize the MIP particles, prepared through the reaction between methacrylic acid monomers and trimethylolpropane trimethacrylate crosslinkers in the presence of morphin, on indium tin oxide (ITO) glass [441]. Another microfluidic amperometric MIP-based morphin sensing system, using 3,4-ethylenedioxythiophene monomers, has also been reported in the literature [439].

Ozcan *et al.* described the development of a pencil graphite electrode modified with an imprinted polypyrrole for the detemination of ascorbic acid. The device was prepared through the electropolymerization of pyrrole monomers onto the electrode by cyclic voltammetry. The electrode was next used in differential pulse voltammetric measurements and showed a respone linearity range and detection limit of 0.25–7.0 mM and 7.4×10^{-5} M respectively [425]. Prasad *et al.* later reported an ascorbic acid sensor based on modification of a hanging mercury drop electrode (HMDE) with MIPs and its use for determinations in the blood serum and pharmaceutical samples. The MIP, which was prepared from melamine and chloranil, was directly coated onto the surface of the HMDE at +0.4 V (vs Ag/AgCl) giving it selectivity towards the template. The method has linear response in the range of 9.80–61.40 μg mL^{-1} and had a detection limit of 0.26 ng mL^{-1} [414].

Ebarvia *et al.* reported two piezoelectric quartz sensors for caffeine based on MIPs [443,447]. In one work they coated a piezoelectric quartz sensor with MIPs templated by caffeine. They used the copolymerization reaction of methacrylic acid and ethylene glycol dimethacrylate monomers, using azobis(isobutyronitrile) as the initiator in chloroform. The sensor response was reported to be linear in the range of 1×10^{-7} mg mL^{-1} and 1×10^{-3} mg mL^{-1} in a stopped flow measurement mode [447]. In another work they used imprinted polymethacrylic acid to the same end, and reported a linear relationship between the frequency shift and the logarithm of the concentration between 1×10^{-9} up to 1 mg/mL and a detection limit of 5.9×10^{-11} mg/mL [443].

Zhou *et al.* reported an MIP optical sensor, as well as a CNT-gold nanoparticle electrode modified with MIPs for chloramphenicol [367,368]. In the former experiment they developed an inverse photonic crystal sensor that could selectively sense

chloramphenicol. They prepared the colloidal crystal template using monodisperse SiO_2 nanosphere and infused the void spaces of the system with precursors of different compositions. Next they removed the SiO_2 and chloramphenicol to yield a molecularly imprinted photonic polymer (MIPP) full of nanocavities derived from the SiO_2. The system had a detection range from 1 ng/mL to 1 mg/mL [367]. The latter effort focused on the development of an electrochemical sensor for chloramphenicol, using MIP to modify MWNTs having carboxilic acid functional groups (c-MWCNT), doped with gold nanoparticles (AuNPs) on the surface of a glassy carbon electrode (MIP/c-MWNTs-AuNPs/GCE). The electrode was reported to possess a linear response in the range of 0.1–100 mg L^{-1} and an LOD of 0.024 mg L^{-1} [368].

In the case of clenbuterol, two sensors have been developed based on MIPs. The first report was on a flow chemiluminescence sensor based on the enrichment of nanogram amounts of clenbuterol by an MIP (methacrylic acid functional monomer, ethylene glycol dimethacrylate crosslinker), and the chemiluminescence reaction between potassium permanganate and formaldehyde in polyphosphate enhanced by clenbuterol. The system revealed linear response in the range of 1.0×10^{-9} g/mL to 5.0×10^{-8} g/mL with a detection limit of 3.0×10^{-10} g/mL [434]. The other report covered an MIP-based potentiometric sensor based on the same MIP, which was reported to exhibit a Nernstian response in the rather wider, and yet higher range of 1.0×10^{-7} M to 1.0×10^{-4} M with an LOD of 7.0×10^{-8} M [377].

Hu *et al.* reported an electrochemical sensor based on self-assembly MWCNTs-clindamycin molecularly imprinted sol-gel with a linear response ranging from 5.0×10^{-7} to 8.0×10^{-5} M, and the LOD of 2.44×10^{-8} M, and its application to the determination of clindamycin in human urine [422]. In another work, Zhang *et al.* have recently reported an assembly of molecularly imprinted sol-gel on an MWCNT modified gold electrode for the selective recognition of the same. The sensor showed a linear reponse towads the concentration of the analyte in almost the same range as the previous work (i.e., 5.0×10^{-7} M to 8.0×10^{-5} M), and the same limit of detection (i.e., 2.44×10^{-8} M) [393].

Prasad *et al.* lately used a layer-by-layer assembled MIP-modified silver electrode for inducing enantiodifferentiation, and hence enantioselective detection of D- and L-thyroxine (using d- or l- thyroxine as the template, 1, 3 diacryl urea as the monomer, and ethylene glycol dimethacrylate), through differential pulse anodic stripping voltammetry and chronocoulometry, and reported detection limits of 0.0060 ng mL^{-1} for L- and 0.0062 for D-thyroxine [397]. Almost simultaneously they reported the enantioselective recognition of D- and L-tryptophan using an imprinted polymer-carbon composite fiber sensor. They used tryptophan as the template, 4-nitrophenylacrylate as the monomer, ethylene glycol dimethacrylate as the crosslinker and 2,2'-azobisisobutyronitrile as the initiator. The results showed the LOD for L-tryptophan to be as low as 0.24 ng mL^{-1} [400].

Roche *et al.* developed a surface plasmon resonance sensor for dextromethorphan based on a molecularly imprinted beta-cyclodextrin polymer with a detection limit of 0.035 μM and a dynamic range of 0.035 μM to 6.00 mM, depending on the instrumental setup [412]. Recently Al-Mustafa *et al.* have reported liquid selective electrodes for dextromethorphan, based on liquid membranes or graphite electrodes coated with polymers of acrylic acid and 2-vinyl pyridine functional monomers and ethylene

dimethacrylate as the crosslinker. They reported the graphite electrodes to have a sub-Nernstian response of 52.4 mV/decade in the range of 5.0×10^{-7} to 1.0×10^{-2} M [346].

Zhang *et al.* reported the use of MIP post-microspheres as sensors for dipyridamole using a bulk acoustic wave (BAW) mechanism. The sensor was reported to have linear response in the range of 4×10^{-8} to 1×10^{-4} M and an LOD of 4×10^{-9} M. TEM studies revealed that there exists a cavity with an average diameter of 150 nm in each post-microsphere. It was mentioned that this kind of post-microsphere will find its application in many aspects [445]. In 2013, Javanbakht *et al.* developed a selective and sensitive MIP-modified carbon paste electrode for dipyridamole using differential pulse adsorptive stripping voltammetry (DPASV) and recorded a linear response in the range of 1.0–110 ng mL^{-1} and an LOD of 0.05 ng mL^{-1} under optimal conditions [356].

The MIP-based epinephrine sensors reported so far include the two reports on flow injection chemiluminescence determination of epinephrine (LR of 5.0×10^{-9}–1.0×10^{-7} M and LOD of 3×10^{-9} M) [448], and a chemiluminescence sensor based on graphene oxide-magnetite-MIPs (LR of 1.04×10^{-7}–7.06×10^{-3} M, and LOD of $1.09 \times 10^{(-9)}$ M) [371]. Heparin sensors based on MIPs have also been reported [348,354] based on the application of a heparin-selective MIP as the recognition element in an indium tin oxide (ITO) electrode [348] or an MIP-based potentiometric sensor [354].

Reports on MIP sensors for lamotrigine include the work of Gholivand *et al.,* who used an MIP-carbon paste electrode and recorded two linear ranges of 0.8–25 and 25–400 nM and the detection limit of 0.21 nM [362]; and that of Sadeghi *et al.*, who used a potentiometric sensor based on an MIP. The latter work revealed a response linearity range of 1×10^{-6} to 1×10^{-3} M and an LOD of 8×10^{-7} M [384].

Gomez-Caballero *et al.* reported voltammetric microsensors for paracetamol based on carbon fiber microelectrodes modified with MIP using square wave voltammetric detection [442]. The MIP was prepared through the electrocopolymerization of o-phenylenediamine and aniline in the presence of paracetamol. The electropolymerization reaction was executed through the use of cyclic voltammetry. The resuting electrode showed a linear response in the range of 6.5×10^{-6} to 2.0×10^{-3} M. In another work, Ozcan *et al.* modified a pencil graphite electrode with imprinted polypyrrole (through cyclic voltammetric deposition of the MIP) and used the device for later differential pulse voltammetry. The results revealed two linear ranges of 5 μM–0.50 mM and 1.25–4.5 mM and a detection limit of 7.9×10^{-7} M [434].

Promethazine sensing devices based on MIPs include an MIP-based potentiometric sensor which was reported to be applicable in the concentration range of 5.0×10^{-7} – 1.0×10^{-1} M, with an LOD 1.0×10^{-7} M [409]; and an MIP-modified carbon phase electrode with two linear response ranges of 4×10^{-12} – 1×10^{-10} M and 1×10^{-9} – 1×10^{-7} M and a detection limit of 2.8×10^{-12} M [381]. Propranolol detection has also been reported through MIP-based phosphorescent probes using tetrabromobisphenol A and diphenylmethane 4,4′-diisocyanate as functional monomers in tetrahydrofuran [380], and an MIP-based ion-selective sensor with a narrow linearity range of 10^{-4}–10^{-5} M [360].

The first report on an MIP-based salbutamol chemiluminescence sensing (MIP-CL) device is the work of Zhou *et al.* on a flow-through chemiluminescence sensor (LR: 5.0×10^{-8}–1.0×10^{-5} g/mL and DL: 1.6×10^{-8} g/mL) [437], which was followed by the more recent work of Qi *et al.* on an MIP-modified CNT electrode, in which case

a poly(o-phenylenediamine) film was deposited on single-walled carbon nanotubes through cyclic voltammetric to prepare the electrode. The linear amperometric response of the device towards the analyte was reported to be in the range of $7.94 \times 10^{-8} - 1.36 \times 10^{-5}$ M with an LOD of 6.08×10^{-8} mol/L [386].

MIP-based tramadol sensing devices include the highly selective MWCNTs carbon paste electrode modified with molecularly imprinted polymers with differential pulse voltammetric detection. The device had a linear response range of 10^{-7} to 10^{-3}M [350]. The other report was on an MIP-electrochemical sensor prepared by coating SiO_2, Fe_3O_4, as the core and the supporting material, with an MIP based on ethyleneglycol dimethacrylate as the crosslinker and functional monomers. The MIP-modified particles prepared this way were eventually incorporated into the modified carbon paste electrode, which was used for cyclicvoltammetry of the analyte within a linear range of 0.01–20 μmol L^{-1} [366].

So far, pharmaceutical compounds with more than one MIP-based sensing device were covered. The following lines will include the target species which have been the subject of fewer investigations. For the ease of access, the cases are sorted in alphabetical order.

A report on an MIP-based 2-aminopyridine (2-AP) by Najafi *et al.* describes a potentiometric sensor for the species containing 2-APH^+ as the active component. The nature of the analyte necessitates the determination to be performed in a bufferred medium at a pH of 4.5, where the majority of 2-AP is protonated to form 2-APH^+. The sensor was reported to have a sub-Nernstian response and detection limit of 54.1 mV/decade and 2.0 mu M, and showed good selectivity to the analyte in the presence of piroxicam impurity [388].

An acetazolamide detection method based on its extraction from plasma and further voltammetric determination, where the MIP was computationally designed for the selective extraction of this analyte from human plasma, was reported. The polymer was composed of acrylamide functional monomers and ethylene glycol dimethacrylate as the crosslinker, and the post extraction pulse voltametric analyses revealed a linearity range and detection limit of 0.20–18.0 μgmL^{-1} and 0.06 μgmL^{-1} [405].

Determination of acetylsalicylic acid based on an electrochemical sensor based on MIP films, which were prepared through copolymerization of p-aminothiophenol and $HAuCl_4$ on an Au electrode surface, is another instance of MIP-based sensing devices. After adding the acetylsalicylic acid template, hydrogen bonds were formed between the amino group of p-ATP and an oxygen of the acetylsalicylic acid. The sensor was used for voltametric analysis and revealed a linear relationship between the current and concentration logarithm in the two ranges of 1 nmol L^{-1} to 0.1 mu mol L^{-1} and 0.7 mu mol L^{-1} to 0.1 mmol L^{-1}, with an LOD of 0.3 nmol L^{-1} [382].

A molecularly imprinted polymer of bis(2,2'-bithienyl)methanes has recently been reported for the selective differential pulse voltametric (DPV), piezoelectric microgravimetric (PM) and capacitive impedometric (CI) determination of adrenaline. To this end, protonated adrenaline was used as the template in the preparation of a thin film of an MIP, which was coating the electrode. The device was later used for the analysis of the template, with detection limits of 2 nM, 0.5 μM and 1.5 μM for the DPV, PM, and CI respectively [357].

In 2009, Lakshmi *et al.* reported a catalytic molecularly imprinted polymer-conducting polymer (MIP-CP) hybrid as an electrochemical element for the determination of catechol and dopamine through chronoamperometry. The application of the CP was to overcome the conductivity complications at the MIP-electrode interface. The CP films were coated on a gold coated glass electrode through electropolymerization of an aniline moiety and the MIP film was next coated thereon through its photochemical grafting by N,N'-diethyldithiocarbamic acid benzyl ester to the polyaniline CP, via activation of the methacrylamide groups. The response was reported to be linear in the range of 228 nM –144 μM and the method showed a detection limit of 228 nM [419].

Javanbakht *et al.* reported an MIP-based potentiometric sensor for cetirizine [429]. The MIP was based on cetirizine dihydrochloride template, methacrylic acid functional monomer and ethylene glycol dimethacrylate crosslinker and was prepared using 2,2'-azobisisobutyronitrile initiator in chloroform. The potentiometric sensor showed a linearity range and detection limit of 1.0×10^{-6}–1.0×10^{-2} M and 7.0×10^{-7} M respectively [429].

Recognition and determination of chlortetracycline based on MIPs using a group of MIP particles has also been reported. The device was reported to possess a Nernstian behavior with the response slopes of 62.5 and 68.6 mV/decade and detection limits of 4.1×10^{-5} and 5.5×10^{-5} M [390].

Huy *et al.* have recently developed an MIP-based optical sensor for clenbuterol and melamine using CdTe quantum dots (QDs) with MIP cappings and used it in the determination of the mentioned species. The preparation of the MIP-CdTe QDs was through a radical polymerization reaction in a mixture of the QDs, the template, 3-aminopropyltriethoxysilane and tetraethoxysilane under controlled reaction regimes. The size of the MIP-CdTe particles was controlled by means of the speed of polymerization, concentration of the template, concentration of the quantum dots, and the ratio of template, monomer and crosslinker. The LODs for clenbuterol and melamine were stated to be 0.4 and 0.6 μM [344]. Nguyen *et al.* introduced another MIP-based optical fiber sensor for cocaine by covalently connecting the MIPs to the distal end of an optical fiber and reported the sensor to show fluorescence increase as the response to cocaine concentration between 0–500 μM [373].

Lu *et al.* reported an MIP-coated SiO_2-based flow injection chemiluminescence sensor for dapsone. The SiO_2 particles were initially modified through grafting amino groups by (3-aminopropyl)triethoxysilane to enhance the cohesion of the MIP layer to the particles. The MIP-coated particles were used for flow injection chemiluminescence analysis of the analyte in the linear range of 1.0×10^{-6}–1.0×10^{-4} M with a detection limit of 5.27×10^{-7} M [375].

Liu *et al.* developed an MIP-based conductimetric sensor for diazepam and achived LOD of 0.008 mg L^{-1} [404]. In another report, MIPs based on doxazosin mesylate template, methacrylic acid monomers, and triallyl isocyanurate as the crosslinkers were prepared and used for the spectrophotometric detection of doxazosin [440]. There have also been reports on an MIP-based potentiometric sensor for doxycycline antibiotic [389] or on MIP-flow injection chemiluminescence determination of doxycycline hydrochloride in the range of 9.0×10^{-7}–6.0×10^{-5} g.mL^{-1} and the detection limit of 3.2×10^{-7} g.mL^{-1} [410].

An (-)-Ephedrine voltammetric sensor prepared through the immobilization of an MIP imprinted polymer for ephedrine in the range of 0.5–3 mM [426], and the MIP-coated gold electrode modified by chitosan-platinum nanoparticles (CS-PtNPs) and graphene-gold nanoparticles (GR-AuNPs) nanocomposites for the determination of erythromycin are other examples of the application of MIPs for the analysis of pharmaceutical species. The latter report used erythromycin as template and 2-mercaptonicotinic acid functional monomers and yielded a linear response in the range of 7.0×10^{-8}–9.0×10^{-5} M and a detection limit of 2.3×10^{-8} M [378].

An MIP-based potentiometric sensor for hydroxyzine with a linearirty range and detection limit of 1.0×10^{-6} to 1.0×10^{-1} M and 7.0×10^{-7} M was reported by Javanbakht *et al.* [428]. Kan *et al.* reported that core-shell MIPs coated on the surface of silica nanospheres, which were initially modified by grafting vinyl groups, directed the copolymerization of the MIP. The resulting nanospheres were incorporated onto a glassy carbon electrode surface and were successfully applied to the determination of hydroxyzine in the linear range from 2.0×10^{-6} to 1.0×10^{-4} M [421].

Prasad *et al.* coated MWCNT surface with inprinted sites using ((p-acryloylaminophenyl)-{(4-aminophenyl)-diethyl ammonium}-ethylphosphate) as the monomer and ethylene glycol dimethacrylate as the crosslinker for the electrochemical detection of insulin and achieved detection limits of 0.0186 nM [396].

In the case of isoniazid, a flow-injection chemiluminescence sensor was developed and a linear range and detection limit of 2×10^{-9}–2×10^{-7} g/mL and 7×10^{-10} M were reported [431]. There is also a single report on a potentiometric levamisole hydrochloride sensor with a linearity range and detection limit of 2.5 μM–100 mM and 1.0 μM respectively [433].

Other reports on the analytical applications of MIPs include potentiometric sensors for (LR:10^{-5}–10^{-1} M DL: 6.0×10^{-6} M) [363]; MIP-modified differential pulse voltammetry carbon paste electrode for methadone (LR: 10^{-7}–10^{-2} M, DL: 10^{-8} M [351], MIP-modified enantioselective piezoelectric quartz crystal sensor for d-methamphetamine (LR: 10^{-5}–10^{-1} μg mL^{-1}, DL: 11.9 pg mL^{-1}) [408]; MIP-PVC graphite electrode for metoprolol (LR: 2.0×10^{-7}–8.0×10^{-3} M and DL: 1.26×10^{-7} M) [394]; MIP-voltammetric sensor for metronidazole voltammetric sensor (LR: 5.64×10^{-5}–2.63×10^{-3} mg L^{-1} and 2.63×10^{-3}–7.69×10^{-2} mg L^{-1}, and DL: 3.59×10^{-5} mg L^{-1}) [391].

Further instances include; TiO_2-modified electrodes coated with 3,4-ethylenedioxythiophene MIP used for the amperometric detection of nicotine (LR: 0–5 mM, DL: 4.9 mu M.) [411]; norfloxacin-Zn(II) MIPs for spectrometric detection of norfloxacin [358]; and MIP-based potentiometric measurement of oxytetracycline (LR: 5.0×10^{-5}–1.0×10^{-2} M, and DL: 19.8 μg/mL) [403].

Benito-Peña *et al.* evaluated six MIPs with penicillin G templates using radioactive and fluorescence competitive assays. They reported pyrenemethylacetamidopenicillanic acid as the tagged antibiotic providing the highest selectivity when competing with penicillin G for the specific binding sites in the MIP prepared with methacrylic acid and trimethylolpropane trimethacrylate. The linearity range for the responses of the method was in the range of 3–890 μM [436].

Zhang *et al.* reported the application of MIP-chemiluminescence for the analysis of phenacetin using $KMnO_4$-phenacetin-formaldehyde reaction as the detection system.

The linearity range and detection limit of the method were reported as 5.0×10^{-7}–5.0×10^{-5} g/mL and 2×10^{-7} g/mL respectively [438].

In another application of MIPs, Peng *et al.* devised a biomimetic bulk acoustic wave (BAW) sensor based on ab MIP as a non-aqueous assay for phenobarbital. The MIP, which was based on phenobarbital template and methacrylic functional monomer, was prepared through a noncovalent method, and the resulting sensor was found to work selectively and sensitively in absolute ethanol (linearity range of the response was in the range of 9.0×10^{-8} M and 5.0×10^{-5} M and a detection limit of 5.0×10^{-8} M was observed [452].

Gholivand *et al.* have recently developed a voltammetric sensor for propylparaben in cosmetics based on a nanosized MIP-carbon paste electrode, which was used in differential pulse voltammetric. A linear range and detection limit of 1–100 nM and 0.32 nM were observed respectively [345].

Peng *et al.* described another MIP-based bulk acoustic wave (BAW) sensor for pyrimethamine based on methacrylic acid functional monomer and ethylene glycol dimethacrylate crosslinker, and recorded a linearity range of 6.0×10^{-7}–1.0×10^{-4} M [451]. There has also been a report on an MIP-film based UV spectrometric sensor prepared with functional abietic-type acids crosslinkers, which was claimed to show highly sensitive responses for quinine in the range of 8.0×10^{-7} to 2.6×10^{-4} M (LOD of 2.0×10^{-8} M) [376]. Arvand and Fallahi devised a noncovalently synthesized MIP-CPE for the differential pulse voltammetry of rivastigmine in the linear range of 2.0–1000 timol L^{-1}, with a detection limit of 0.44 timol L^{-1} [364].

Molecularly imprinted polymer-modified electrodes for the amperometric detection of salicylic acid (LR: 6×10^{-5}–1×10^{-4} M and DL: 2×10^{-5} M) [420]; MIP membranes for the potentiometric determination of sertraline (LR: 1.0 μM–10 mM DL: 0.8 μM and a near Nernstian response of 57.7 mV decade^{-1}) [379]; a surface plasmon resonance (SPR) sensor for sialic acid based on MIPs prepared through copolymerizing N,N,N-trimethylaminoethyl methacrylate, 2-hydroxyethyl methacrylate and ethyleneglycol dimethacrylate in the presence of p-vinylbenzeneboronic acid ester with sialic acid [450]; MIPs designed for the recognition of chiral (S)-ibuprofen through IR and UV-Vis spectrometry [359]; biosensors for the determination of sorbitol based on the electropolymerization of o-phenylenediamine (o-PD) on quartz crystal (LR: 115 mM and DL:1 mM) [446]; flow-injection analysis of sulfamethoxazole using hemiluminescence and chitosan/graphene oxide MIPs (LR: 1.0×10^{-7}–2.3×10^{-3} M and DL: 2.9×10^{-8} M) [352]; MIP-based potentiometric recognition of sulfamethoxazole (LR: 6.0×10^{-8}–3.1×10^{-3} M and DL: 3.5×10^{-9} M) [365]; and an MIP-modified carbon paste electrode for the differential pulse voltammetry of sulfasalazine (LR: 1.0×10^{-8}–1.0×10^{-6} M and DL: 4.6×10^{-9} M) [370], are other cases of applications of molecularly imprinted polymers in the analysis of pharmaceuticals.

Other instances include a micro-flow sensor on a chip for the analysis of terbutaline through chemiluminescence (LR: 8.0–100 ng/mL and DL: 4.0 ng/mL) [435]; an MIP-based electrochemical sensor for the impedance spectroscopic and chronoamperometric determination of theophylline (LR: 2.00×10^{-7}–3.45×10^{-4} M and DL: 1.00×10^{-7} M) [369]; a capacitive sensor for cyclic voltammetry of thiopental (LR: 3–20

μM and DL: 0.6 μM) [374]; an MIP-based amperometric sensor for triclosan based on the electropolymerization of o-phenylenediamine functional monomers (LR: 2.0×10^{-7}–3.0×10^{-6} M and DL: 8.0×10^{-8} M) [417]; potentiometric determination of trimethoprim through selective MIP-coated graphite electrodes using methacrylic acid and 2-vinyl pyridine as monomers (DL: 4.01×10^{-7} M) [385]; an MIP-modified optode for the selective determination of vancomycin (LR: 10–700 μM) [343]; and finally, imprinted poly(o-phenylenediamine-co-aniline) electrodes for the analysis of warfarin in human samples through differential pulse voltammetry (LR: 10^{-6}–10^{-4} M and DL: 6.22×10^{-8}) [402].

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10

Biobased Pharmaceutical Polymer Nanocomposite: Synthesis, Chemistry and Antifungal Study

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Abstract

Solvent-free, microwave-assisted synthesis of bionanocomposite (poly(ester-ricinoleamide) (PERA)/ nano ZnO) (*in-situ* synthesized nano ZnO) was carried out by the utilization of *Ricinus communis* or Castor oil, complying with the principles of "Green Chemistry." Fourier transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM) techniques confirmed the formation of bionanocomposites. Antifungal activity was examined by MIC_{90}, disc diffusion assay, growth curve study and proton extrusion measurements. Growth of organisms on solid and liquid media was significantly affected by synthesized compounds at varying concentrations. We also investigated their effect on plasma membrane mediated H^+ pumping by various *Candida* species. Tentative mechanism of action appears to originate from inhibition of plasma membrane ATPase activity that ultimately leads to intracellular acidification and cell death.

Keywords: Polyesteramide, zinc oxide, castor oil, antifungal, microwave-assisted synthesis, plasma membrane H^+-ATPase, *Candida*

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10.1 Introduction

Fungi have emerged worldwide as an increasingly frequent cause of infections posing serious nosocomial threats to patient populations. Incidences of candidiasis have increased significantly in recent years owing to HIV infection, prolonged antibiotic therapy, organ transplants and cancers. Although the infections caused by non-albicans *Candida* species have increased, *Candida albicans* is still the most common fungal pathogen and has been established as the predominant cause of virtually all types of candidiasis. At present there are seven different classes of antifungal drugs that are used to treat fungal infections, viz. polyenes, pyrimidine analogues, azoles, echinocandins, allylamines, thiocarbamates and morpholines. Among all of them, only azoles are currently the most widely used class of antifungals. They act by preventing the synthesis of ergosterol, a major component of fungal cell membranes, by inhibiting the enzyme 14 α-lanosterol demethylase, altering membrane fluidity, reducing activity of membrane-associated enzymes and increasing permeability. Among azoles, for example, fluconazole, which is highly toxic to human body and leads to resistance during therapy, is still used to cure fungal infections. At present the nephrotoxic effects and well-known toxicity of amphotericin B to the host cell definitely requires discontinuation of its therapy. It is the selective pressure of these drugs that have made most of the fungal species resistant. Treatment options for invasive infections are limited and almost always involve the use of nephrotoxic amphotericin B and azoles, basically fluconazole, which leads to resistance on prolong use [1–11]. Such serious global health problems demand a renewed effort seeking the development of new antimicrobial agents effective against different fungal species resistant to present day antifungals. At present, we need to search for a non-antibiotic agent that is highly effective and much safer for the eradication of both susceptible and resistant fungal species. Biobased resources such as plant oils derived from clove, coconut, cinnamon, tulsi, lemongrass, tea tree, olive, and others are gaining much attention for the treatment of fungal infections [11–15].

In the present scenario, attention has been given to materials synthesized from renewable or biobased resources such as vegetable seed oils (VO), polysaccharides (mainly cellulose and starch), sugars, wood, and others for the substitute of commercial materials derived from petroleum resources. Among them vegetable seed oils are potential candidates to this end owing to the varied functional attributes they posses. They have been utilized for making a number of environmentally friendly, low molecular weight polymeric materials, and are also expected to be biodegradable, nontoxic and biocompatible, which collectively enhances their application in biomedical sciences as biodegradable materials [12–29]. Conventional synthesis of biobased polymers and composites is complex, multistep, energy and time consuming. Nowadays, "Green Chemistry" calls for the development of eco-friendly polymeric materials having desirable mechanical properties**.** Green Chemistry Revolution has provided an alternate mode for chemists and industrialists to develop polymers via an eco-friendly route, to minimize the utilization of toxic chemicals, which are hazardous to health and environment. Green technologies involve bio-based polymers as well as synthesis of these polymers and composites through Green Chemistry [19,20,28]. Therefore, the present work reports the development of an environmentally friendly

material, bio-based poly(ester-ricinoleamide) (PERA) (obtained from oil of *Ricinus communis*(RCO) or Castor from its seeds) and bionanocomposites (PERA/nano ZnO) derived from it by simple, energy and time efficient method (microwave-assisted synthesis). PERA is a polymer that shows the properties of both polyesters and polyamides. It is generally prepared by two steps: (i) transformation of RCO to N,N-bis(2 hydroxyethyl)ricinoleamide by base catalyzed amidation reaction, and (ii) by esterification reaction of the latter with an acid or anhydride.

In this work, nano-ZnO particles were synthesized *in situ* during the polymerization reaction of PERA. Spectral (FTIR) and morphological studies (optical and transmission electron microscopy [TEM]) were used to confirm the formation of PERA/nano ZnO. The abilities of these synthesized materials to kill *Candida* cells were tested and identified by minimum inhibitory concentration (MIC_{90}), disc diffusion assay and growth studies. The main objective of this study was to further elucidate the antimicrobial mechanism of action of PERA and PERA/nano ZnO by determining the effect on the activity of H^+ATPase located in membranes of pathogenic *Candida* species. This work is an additional effort to the development of new chemotherapeutic treatments of infections by using plant-derived materials which are fungicidal, biodegradable and prevent drug resistance.

Such bionanocomposites may pave way towards Green Pharmaceutical Chemistry considering the raw materials being derived from natural resource, synthesis being accomplished via Green Chemistry route, and being biodegradable in nature. These may serve for topical or transdermal applications in fungal infections and wound care.

In the present chapter, an attempt has been made to investigate the antifungal behavior of novel nanocomposite material chemically derived *in situ* from VO-based polymer-PERA by microwave-assisted preparation method. This interesting approach is the first of its kind to the best of our knowledge. It deals with energy-efficient, time saving chemical transformation of a VO into polymer/metal oxide nanocomposite, the latter bearing antifungal properties. However, before gaining insight into this, it is mandatory to gain knowledge about the structure and properties of VO, VO-derived polyesteramides (PEAs), metal oxide nanoparticles, "Green Chemistry" and microwave-assisted reactions.

10.1.1 Vegetable Seed Oils(VO)

The forseeable exhaustion of petroleum reserves by 21st century has stimulated fortified interests in research pertaining to identifying and fostering alternative renewable precursors for the production of polymer materials. Efforts are in full swing to introduce chemicals from biomass into general preparation of green polymers. In the present scenario, the consumer and industrial interests in the development of ecofriendly materials have catapulted the environmentally benign agricultural resources as the feedstocks of the polymer industry. Today, due to interdisciplinary approaches through research and technological innovations in chemistry, the biosciences, biotechnology and engineering, it is possible to design specialty chemicals from nature's abundant renewable resources such as starch, lignin, protein, cellulose, chitosan, shellac, rosin, polyhydroxyalkanoates, furanone, alginate, wool fibers and VO for innumerable

industrial applications such as plasticizers, biodiesel, lubricants, adhesives, packaging materials, printing inks, paints and coatings, antimicrobials, cosmetics, pharmaceuticals, and others. Amongst these, VO are nature's nontoxic, nondepletable, domestically abundant, nonvolatile and biodegradable resources, yielding polymers capable of competing with fossil fuel-derived petro-based products. VO comprise one of the most important components of biomass. The oil seeds are cheaper, easily and abundantly available, oil extraction techniques are not very cumbersome and equipment for the same is affordable. VO hold superiority in terms of cost, availability and yield, over other components of biomass. They have been used for centuries as chief ingredient in polymers due to their functional attributes. VO yield several low molecular weight materials that find versatile applications [16–18].

Chemically, VO are triesters of glycerol and fatty acids such as oleic, linoleic, linolenic, palmitic, and others. The difference in fatty acid composition, such as variations in functional groups, fatty acid chain lengths, position of double bonds and others, offer varying properties to VO and their respective derivatives. Often, unusual fatty acids such as petroselenic, erucic, vernolic, ricinoleic and others are also present. Thus, VO hold immense scope for transformation into platform chemicals for versatile applications. VO mainly consist of triglycerides as major (93 wt% – 98 wt%) and diglycerides, monoglycerides and phosphoglycerides as minor components. The term "triglyceride" refers to triesters of glycerol, while the term "oil" is meant for those triglycerides that are liquids at room temperature. A few examples of fatty acids are saturated fatty acids such as stearic, arachidic, ehenic, lignoceric acids; in addition to common saturated fatty acids such as oleic, linoleic and linolenic fatty acids, other unsaturated fatty acids such as elaidic, palmitoleic, myristoleic, petroselenic, linoleladic, α-eleostearic, β-eleostearic, ricinoleic, lauric acids are also present. Fatty acids bearing oxirane ring (*Vernonia* [VRO], *Euphorbia* and *Cephalocroton* oils), hydroxyl functionalities (*Ricinus communis* or *Castor* [CO], *Strophantus, Lesquerella* [LSO], *Coriaria, Cardamine impatiens, Sebastiana commersoniana* oil) and furanoid fatty acids (*Exocarpus cupressiformis, Hevea brasiliensis)* are also found. The characteristic nature of every oil is governed by its fatty acid profile, which varies by local weather conditions, soil, growth conditions of crops and purification methods, causing variation in fatty acid composition, among same or different types of oil species. The properties of VO derivatives are governed by fatty acid composition of starting oils, that is, stereochemistry of double bonds of fatty acid chains, their degree of unsaturation and length of carbon chains of fatty acids [16–21]. Based on the orientation of hydrogen atoms of double bonds, fatty acids are classified as *cis* or *trans*. Naturally occurring fatty acids generally have cis configuration. Considering their degree of unsaturation, described by their iodine value [IV]), as well as their Drying Index [DI] {DI=linoleic%+(2linolenic%)}, oils are classified as "drying" (IV>130; DI>70), "semi-drying" (115<IV<130; DI between 65 and 75) and "non-drying" (IV<115; DI< 65) as in linseed (LO), soybean (SO) and palm kernel oil, respectively. VO are abode to several important functionalities and active sites such as hydroxyls, oxiranes, double bonds, allylic carbons, esters, alpha carbon to the ester group and others, amenable to host of chemical reactions leading to synthesis of several monomers and polymers; some of these reactions and important derivatives have been discussed in proceeding sections. By employing basic principles of chemistry, as in polymers derived from petrochemicals, active sites in oils

may be harnessed to introduce polymerizable groups into triglyceride backbone. It is worth mentioning that of all oleochemical reactions, 90% involve modifications at carboxyl functionalities, while the rest involve double bond reactions [16–28]. VO or naturally available triglycerides may be converted into epoxies and hydroxyls by chemical reactions at their double bonds. The common reactions involve epoxidation, hydroformylation, ozonolysis, hydrogenation, metathesis and others [22,27,28]. Another pathway includes the conversion of VO by chemical reactions at carboxyl functionalities into monoglycerides and amides, through glycerolysis, and amidation, which can further be transformed into polymeric resins such as alkyds, polyesters, polesteramides, polyetheramides by reaction with diacids, dianhydrides or alcohols, finding enormous applications in field of coatings and paints, antibacterial and antifungal agents, drug delivery, and others [16].

10.1.2 Polyesteramides (PEAs)

PEAs are amide-modified esters. They can be used at temperature up to 175°C and have excellent resistance to thermal aging and good mechanical resistance. They can be used as electrical insulators and hot melt adhesives. PEAs can be categorized as alternating, non-alternating and block copolymers. In these categories, a variety of PEAs such as unsaturated or saturated, aliphatic-aliphatic, fully aromatic, aliphatic-aromatic have been synthesized by using interfacial, solution and melt polymerization. In most of them, alternating polyesteramides (AltPEA) might have a high crystallinity and high modulus. These combine good properties of polyester and polyamide, such as high melting temperature, fast crystallization, good mechanical properties, good solvent resistance and low water absorption, and find applications for both engineering plastics and thermoplastic elastomers. AltPEA are usually prepared by a two-step synthesis. Firstly, well-defined monomers are prepared which react in a second step. In this way a polymer with a regular structure is obtained. One-step synthesis in which all the monomers are reacted at the same time usually yields a polymer of low order. The irregular structure hinders the ordering in a crystalline structure. Gaymans *et al.* have described the synthesis, melting behavior, crystalline structure and some mechanical properties of segmented PEAs copolymer with short, partially aromatic esteramide units of uniform length and segment. They have also described the tensile properties of segmented copoly(ether-esteramide). Many PEAs have been found to show liquid crystallinity. Many papers and patents are available which describe the synthesis and properties such as elemental analysis, FTIR, ^{13}C CP/MAS NMR, solubility, viscosity, DSC, WAXD, polarized optical microscopy and TGA of thermotropic and thermoplastic polyesteramide having liquid crystallinity. These polymers have unique thermomechanical properties. Several thermotropic liquid crystalline poly(amide ester)s containing flexibile spacers were also prepared [30–35]. Petro-based polyesteramides have high thermal stability. They are mainly thermoplastic in nature and have high molecular weight as well as high melting point, lower intractability and lower solubility, which inhibit their use as coating material. These drawbacks are overcome by development of VO-based PEAs.

VO-based PEAs are amide/amino-modified alkyds and are also thermosetting in nature. They possess both ester (-COOR) and amide (-CONRR') linkages in one

polymer chain. The presence of these units improves the characteristics of PEAs over normal alkyds in terms of hardness, ease of drying, water vapor resistance as well as durability and other physicochemical properties. Synthesis of the resin involves two steps as alkyds. The first step is aminolysis, which is synthesis of VO fatty amide (monoglyceride) by reaction of VO and diethanolamine; and the second step is esterification/transesterification between phthalic acid/anhydride and oil fatty amide/monoglyceride.

PEAs synthesized from VO as starting material for coatings have generated considerable interest throughout the world. Several PEAs have been synthesized from conventional and non-conventional VO such as linseed, castor, pilu fat, soybean, *Annona squamosa*, argemone, *Pongamia glabra* and nahar. They possess improved characteristics over normal alkyds in terms of hardness, ease of drying, water vapor resistance, as well as durability and other physicochemical properties. These PEAs have found applications as paint and corrosion protective coatings. Further modifications in PEAs have also been carried out to improve their curing temperature/time or drying ability, and physicomechanical and corrosion protective properties. Such modifications involved the incorporation of metal, styrene, vinyl acetate, acrylic group, urethane linkages, butylated melamine formaldehyde (BMF), and improved their mechanical and corrosion protective efficiency [36–38]. A detailed description of VO-based PEAs is provided in an earlier publication [16]. In this work, an attempt has been made to develop VO-based antifungal PEAs nanocomposites for the first time, via incorporation of ZnO nanoparticles, by their *in-situ* preparation, using functional groups present on polymer chain without using any solvent, capping agent, surfactant or others [6–11].

10.1.3 Zinc Oxide Nanoparticles

The incorporation of nano metal oxides in bioderived polymers towards the formation of bionanocomposites integrates novel properties of both organic and inorganic (very low loading) counterparts in the final product. These have found potential applications in various fields, most importantly in biomedicine. Nano zinc oxide, ZnO, is a nontoxic and environmentally friendly metal oxide that has significant optical, antimicrobial, antibacterial, bactericidal and efficient UV absorption properties along with other important properties. It has potential applications in the field of catalysts, electrical and optical devices, varistors, sensors, solar cells, cosmetics, antimicrobials and so on. The use of metal oxides as antimicrobial agents has the advantage of improved safety and stability, as compared to organic antimicrobial agents [39]. Nano zinc oxide and their nanocomposites (with low loading of nano ZnO) have been widely tested for their antibacterial properties [40–45], whereas scant literature is available on their antifungal activity [39,43,44]. Sawai *et al.* [43] and Gomes *et al.* [45] observed that the micron-sized ZnO particles were unsuitable for control of fungal growth. Fang *et al.* [46] reported more promising results with ZnO (whisker form) against *C. albicans*. In another work by He *et al.* [44], significant antifungal activities and mode of lethal action of ZnO nanoparticles (sizes of 70 ± 15 nm) against postharvest pathogenic fungi was observed by scanning electron microscopy and Raman spectroscopy. Lipovsky *et al.* [41] reported antifungal activity of ZnO nanoparticles (100 nm and 10 nm) against *C. albicans*. Lipovsky *et al.* and He *et al.* both observed concentration- and size-dependent marked cytotoxic effect of ZnO nanoparticles against fungi [41,44–46]. There is

no literature on antifungal activity of renewable resource-derived polymer/nano-ZnO bionanocomposites [47].

The literature has revealed the enormous potential of metal oxide nanoparticles in conferring antimicrobial characteristics. The efficient antimicrobial action of metal nanoparticles is attributed to their large surface area and size/shape-dependent physicochemical properties. Many mechanisms are proposed for antimicrobial action of metal nanoparticles. Nanoparticles may attach to the surface of cell membrane, disturbing permeability and respiration functions of cell [48–50]. The nanoparticles provide solutions to multiple challenges related to microbe associated/generated problems, however, the point of concern is that their synthesis, processing conditions, and applications should be environmentally safe, addressing the principles of Green Chemistry. These principles prescribe minimizing the use of unsafe products and maximizing the efficiency of chemical processes by using environmentally benign solvents and nontoxic chemicals [48].

10.1.4 Green Chemistry

The term "Green Chemistry" (GC) was coined by Paul Anastas in the year 1991. It is the design of chemical products and processes that reduce or eliminate the generation of hazardous substances. The United States Environmental Protection Agency has put efforts into the speedy adoption of this revolutionary and diverse discipline, contributing to significant environmental benefits, innovations and strengthened economy. The principles of GC extend beyond the concerns and hazards of chemical toxicity and also include energy conservation, waste reduction, and life-cycle considerations; for example, the use of more sustainable or renewable feedstocks, designing for end of life or the final disposition of the product. Paul Anastas and John Warner have outlined the following list of principles addressing what would make a greener chemical process, or product [51,52]. These include the following:

1. **Prevention:** *It is better to prevent waste than to treat or clean up waste after it has been created*. This principle is well in line with an old saying "Prevention is better than cure." According to this principle, it is suggested that prevention of waste being created is better than considering remedies towards its treatment or cleaning after it has been created.
2. **Atom Economy:** *Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.*
3. **Less Hazardous Chemical Syntheses:** *Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and environment.* This is an effort to create a clean and green environment for human beings, plant and animal life.
4. **Designing Safer Chemicals**: *Chemical products should be designed to affect their desired functions while minimizing their toxicity.*
5. **Safer Solvents and Auxiliaries**: *The use of auxiliary substances (e.g., solvents, separation agents,) should be made unnecessary wherever possible and innocuous when used.*

6. **Design for Energy Efficiency:** *Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.*
7. **Use of Renewable Feedstocks:** *A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.*
8. **Reduce Derivatives**: *Unnecessary derivatization (use of blocking groups, protection/ deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.*
9. **Catalysis:** *Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.*
10. **Design for Degradation**: *Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.*
11. **Real-time analysis for Pollution Prevention:** *Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.*
12. **Inherently Safer Chemistry for Accident Prevention**: *Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.*

In the present work, we have attempted to comply with the following principles of GC. Firstly, we have used a VO which is an abundantly available renewable feedstock (Principle No. 7). Secondly, we have obviated the use of any solvent, taking advantage of the inherent fluidity of VO polymer, due to long aliphatic chains of parent VO (Principle No. 5). The synthesis was performed in a microwave oven, which may be considered as an energy efficient process, reducing the time period of reaction and giving better yield at the end (Principle No. 6). The synthesis method is not hazardous, employing safer chemistry, and all raw materials are used up in the process producing the final product and are also biodegradable in nature since derived from VO (Principle Nos. 1, 2, 3, 4, 8, 10 and 12) [51–53].

10.1.5 Microwave-Assisted Reactions

Microwaves are electromagnetic radiations with frequencies between 300 GHz and 300 MHz (with a wavelength in the range of 1 mm to 1 m). Most commercial microwave ovens produce a microwave wavelength of 12.25 cm, which corresponds to a frequency of 2.45 GHz (2·45 GHz is mostly used for household microwave ovens and 0·915 GHz is preferred for industrial/commercial microwave ovens). Today, microwaves have gone from our domestic kitchens to commercial catering industries and also research laboratories. They are being used widely in heating materials for industrial and domestic purposes. Microwave irradiations offer several advantages relative to conventional heating.

They provide instantaneous and rapid bulk heating, direct heating, high temperature homogeneity, selective heating and energy savings. These apprise much improved reaction rates, milder reaction conditions and formation of cleaner products, higher yields and minor wastes, as well as reduction of side reactions. During the past decades, microwave irradiation has developed into a highly useful and environmentally benign technique, providing an effective alternative energy pathway for chemical reactions and other processes. Such "specific" microwave effects are a consequence of dielectric heating. In contrast to conventional heating in which the material's surface heats first and then the heat moves inward, in microwave technology, electromagnetic waves pass through material and cause its molecules to oscillate, generating heat. Microwave heating generates heat within the material and heats the entire volume at about the same rate. Microwave technology has got an obvious edge and advantages over conventional methods of heating. As a result of this, microwave-assisted methods are being employed to carry out various synthetic procedures successfully and effectively, which were otherwise encountering problems earlier. Moreover, another feature is the possibility of carrying out reactions without solvent. Such reactions can be carried out in open vessels, thus avoiding risk of development of high pressure in addition to associated ease of manipulation. Furthermore, these reactions reduce the overall costs and lower amounts of byproducts, with minor environmental impact, ensuring high levels of "atom economy" [54–58].

10.2 Experimental Protocol

RCO was procured from local market, New Delhi, India (Mn: 930; hydroxyl value – 160-168 mg KOH/g; acid value – 2.45 mg KOH/g). All the chemicals were purchased from Aldrich (USA) and Merck (Germany). Thin-layer chromatography (TLC) method, acid value and refractive index were used to monitor progress of reactions, which were carried out by standard laboratory methods [59]. Precoated aluminium sheets (silica gel 60 F254, Merck, Germany) were used for TLC and spots were visualized under UV light. The solubility was investigated in xylene, DMSO, alcohol and water by taking 100 mg of test sample in 20 ml of solvent in a closed test tube and set aside for 24 h. FTIR spectra were taken on IRPrestige-21, IRAffinity-1, FTIR-8400S (Shimadzu Corporation Analytical and Measuring Instrument Division, F.R. Germany) using a NaCl cell. The formation of nano ZnO within bionanocomposite is confirmed by TEM (JEOL 2100F, operated at 200 kV) on a carbon-coated copper grid.

10.2.1 Procedure for Transformation of RCO to N,N-bis(2 Hydroxyethyl) Ricinolamide (MicHERA)

Microwave-assisted synthesis of MicHERA was carried out as per reported method [60]. It was obtained as a light yellow color liquid (yield 98–100 %). FTIR (cm^{-1}): 3369.3 (broad –OH), 3010 (υ, C = C–H), 2923(υ, $-CH_2$ asym), 2853 (υ, $-CH_2$ sym), 1636.2 (υ, > C = O amide), 1078.9 cm^{-1} and 1052.9 cm^{-1} (υ, C-OH, primary and secondary).

10.2.2 Procedure for Transformation of MicHERA to PERA/Nano-ZnO Bionanocomposite

PERA/Nano-ZnO bionanocomposite was synthesized in domestic microwave oven model LG MS 1927C operating at 230V- 50 Hz frequency. MicHERA (0.04 mole) and finely powdered divalent zinc acetate (1 wt%, 3 wt%, 5 wt% and 7 wt% of monomer) were taken in an Erlenmeyer flask and placed in a microwave oven for 2–3 min, thereafter finely powdered maleic anhydride (0.04 mole) was added in the same pot and kept again for 2–3 min under same conditions. The reaction was monitored by TLC and acid value. The synthesized bionanocomposites were translucent light yellow color liquids (98–100% yield), designated as PERA/nano ZnO-1, PERA/nano ZnO-3, PERA/nano ZnO-5 and PERA/nano ZnO-7 (suffix indicates the % of divalent zinc acetate; yield 100%). FTIR (cm^{-1}): 3373- 3367- (-OH), 3057-3051 (υ, C=CH- maleic), 3007-3005 (υ, C=C-H of fatty acid alkyl chain), 2926.01(υ, $-CH_2$ asym), 2856.58 (υ, $-CH_2$ sym), 1737-1732 (υ, >C=O maleic), 1625-1618(broad, υ, >C=O amide), 1222-1172 (C–C(=O) –O–C), 1120-1122 (υ , C-O secondary hydroxyl), 1068-1066 (υ, C-O primary hydroxyl), 657-653(δ, surface -OH of nano ZnO), 472-463(υ, nano Zn-O).

10.2.3 Procedure for Transformation of MicHERA to PERA

Virgin PERA was prepared by similar method with MicHERA and maleic anhydride as starting materials under aforementioned conditions (Section 10.2.2). It was obtained as light yellow color liquid (yield 100%). FTIR (cm^{-1}):3442.94 (-OH), 3057.17 (-C=C-H maleic), 3003.17 (υ, C=C–H fatty acid alkyl chain), 2926.01(υ, $-CH_2$ asym), 2856.58 (υ, $-CH_2$ sym),), 1737.86 (υ, > C=O ester), 1651(υ, >C=O amide), 1292-1168 (C–C(=O) –O–C), 1130 (υ, C-O secondary hydroxyl), 1043.49 (υ, C-O primary hydroxyl).

10.2.4 Fungal Isolates Used and Minimum Inhibitory Concentration (MIC_{90}) Determination

Table 10.1 provides list of fungal isolates involved in this study. The isolates were collected from Regional STD (Sexually Transmitted Disease) Centre, Safdarjung Hospital, New Delhi, India. All of the strains were grown on yeast extract, peptone and dextrose (YPD) medium containing 2% (w/v) glucose, 2% peptone, and 1% yeast extract (Hi

Table 10.1 Isolates used in the present study.

Classification of Isolates	Type of Isolate
Sensitive (Clinical, n=09)	
Invasive (n=3)	*C. albicans* (1), *C. krusei* (1), *C. glabrata* (1)
Cutaneous (n=3)	*C. albicans* (1), *C. glabrata* (1), *C. krusei* (1)
Respiratory (n=3)	*C. albicans* (2), *C. tropicalis* (1)

Media, India). YPD agar plates containing 2.5% agar (Hi Media) in addition were used to maintain the culture. All inorganic chemicals were of analytical grade and procured from E. Merck (India). Fluconazole was purchased from Hi Media (India). To determine minimum inhibitory concentration values, the cells were grown for 48 h at 30°C to obtain single colonies, which were resuspended in a 0.9% normal saline solution to give an optical density at 600 nm (OD_{600}) of 0.1. The cells were then diluted 100-fold in YNB medium containing 2% glucose. The diluted cell suspensions were added to the wells of round-bottomed 96-well microtiter plates (100µl/well) containing equal volumes of medium and different concentrations of test agents [61]. A drug-free control was also included. The plates were incubated at 30°C for 24 h. MIC test end point was evaluated both visually and by observing OD_{620} in a microplate reader (BIO-RAD, iMark, US) and is defined as the lowest compound concentration that gave ≥ 90% inhibition of growth compared to the controls.

10.2.5 Disc Diffusion Halo Assays

Strains were inoculated into liquid YPD medium and grown overnight at 37°C. The cells were then pelleted and washed three times with distilled water. Approximately 10^5cells/ml were inoculated in molten agar media at 40°C and poured into 100-mm-diameter petri plates. The test materials initially dissolved in 1% DMSO were further diluted in distilled water to concentration ranges of 10-fold of their respective MICs. 4-mm-diameter sterile filter discs were impregnated with the test compounds as reported previously [61]. 1% DMSO (solvent) and 100 µg/ml of fluconazole were also applied on the discs to serve as negative and positive controls, respectively. The diameter of zone of inhibition was recorded after 48 h and was compared with that of control. The type of halo produced depicts fungicidal/static characteristic of test entity.

10.2.6 Growth Curve Studies

For growth studies, 10^6 cells/ml (optical density $A_{600} = 0.1$) culture of *Candida* cells were inoculated and grown aerobically in YPD broth for control along with varied concentrations of test materials in individual flasks. Growth was recorded turbidometrically at 595 nm using Labomed Inc. Spectrophotometer (USA) as reported previously [62]. The growth rate study of different *Candida* species in absence as well as in presence of inhibitor was performed for each concentration in triplicate, average of which was taken into consideration.

10.2.7 Proton Efflux Measurements

The proton pumping activity of *Candida* species was determined by monitoring acidification of external medium by measuring pH as described previously [63,64]. Briefly, mid-log phase cells harvested from YPD medium were washed twice with distilled water and routinely 0.1 g cells were suspended in 5 ml solution containing 0.1 M KCl, 0.1 mM $CaCl_2$ in distilled water. Suspension was kept in a double-jacketed glass container with constant stirring. The container was connected to a water circulator at 25°C. Initial pH was adjusted to 7.0 using 0.01 M HCl/NaOH. Test materials were added to

achieve the desired concentrations (MIC_{90}) in 5 ml solution. For glucose stimulation experiments, 100 µl of glucose was added to achieve a final concentration of 5 mM in total volume of suspension. H^+ extrusion rate was calculated from the volume of 0.01 N NaOH consumed.

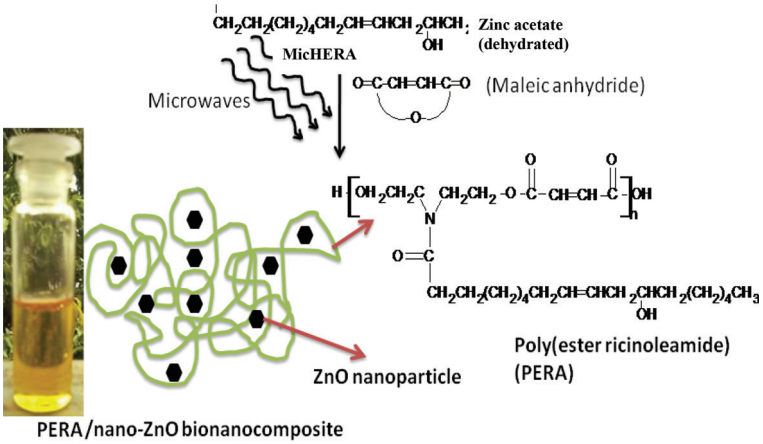
10.2.8 Measurement of Intracellular pH (pHi)

Intracellular pH was measured as performed earlier [65] with modifications. Mid-log phase cells grown in YPD medium were harvested and washed twice with distilled water. Cells (0.1 g) were suspended in 5 ml solution containing 0.1 M KCl and 0.1 mM $CaCl_2$. Desired concentrations of test materials (their respective MIC_{90} values) were added to the suspension and pH was adjusted to 7.0 in each case. Following incubation for 30 min at 37°C with constant shaking at 200 rpm, pH was again adjusted to 7.0. Nystatin (20 µM) dissolved in DMSO was added to the unbuffered cell suspension and incubated at 37°C for 1 h. The change in pH of suspension was followed on pH meter with constant stirring. The value of external pH at which nystatin permeabilization induced no further shift was taken as an estimate of pHi [66].

10.3 Results

10.3.1 Synthesis

PERA/nano-ZnO bionanocomposites were synthesized according to the procedure depicted in Scheme 10.1. Microwave-assisted rapid *in-situ* synthesis of PERA/nano-ZnO bionanocomposite was carried out by the reaction of bio-derived monomer, MicHERA, maleic anhydride and divalent zinc acetate (1 wt%, 3 wt%, 5 wt% and 7 wt% of monomer) under microwave irradiations in domestic microwave oven without



Scheme 10.1 Synthesis of bionanocomposite from oil-derived monomer.

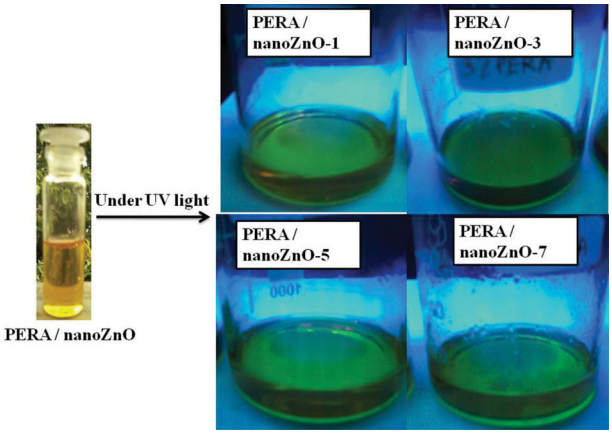


Figure 10.1 Photographs of different composition of PERA/nano-ZnO bionanocomposites show change in color when exposed under UV light.

any volatile organic solvents. The synthesized bionanocomposites were designated as PERA/nano ZnO-1, PERA/nano ZnO-3, PERA/nano ZnO-5 and PERA/nano ZnO-7 (suffix indicates the % of divalent zinc acetate). TLC and acid value were used to monitor the reaction. The prepared bionanocomposites were translucent light yellow color liquid (98–100% yield) that showed an emission of green color when exposed to UV light (Figure 10.1). The bionanocomposites were highly soluble (100%) in alcohol, xylene and DMSO. They were characterized by FTIR (Figure 10.2) and refractive index. TEM analysis was used to confirm *in-situ* synthesis of nano-ZnO particles (sizes 4.0–8.4 nm) within the polymer matrix. PERA served as the matrix, solvent, capping agent, surfactant for *in-situ* synthesis of nanoparticles and also provided functional groups for chemical reaction leading to formation of nano ZnO.

Refractive index of PERA, PERA/nano ZnO-1, PERA/nano ZnO-3, PERA/nano ZnO-5 and PERA/nano ZnO-7 are 1.540, 1.530, 1.524, 1.520 and 1.512, respectively. It reveals that the refractive index of PERA was higher than PERA/nano-ZnO bionanocomposite. The values further decrease as the percentage of metal acetate increases. It can be correlated to decrease in viscosity with the loading of zinc acetate. The solubility results of PERA and PERA/nano ZnO bionanocomposites showed 100% solubility in xylene, DMSO, alcohol, while insoluble in water. Solubility of bionanocomposites could be correlated to homogenous mixing of contents and formation of ZnO in the matrix. The pure zinc acetate was not dissolved in alcohol and xylene, while it dissolved in water. Figure 10.2 shows FTIR spectra of PERA and different compositions of bionanocomposite. It reveals the formation of nano ZnO within PERA matrix. The bionanocomposite was investigated by TEM analysis at different magnifications. Figure 10.3 shows TEM images of PERA/nano ZnO-7. It reveals *in-situ* synthesis of nano-ZnO particles within PERA matrix. They are observed in nano clusters of particles (4.0–8.0 nm) and size of clusters are also in the range of 14.2 nm – 20.0 nm. These clusters of nanoparticles are arranged in PERA matrix in self-assembled manner and form a rod-like structure.

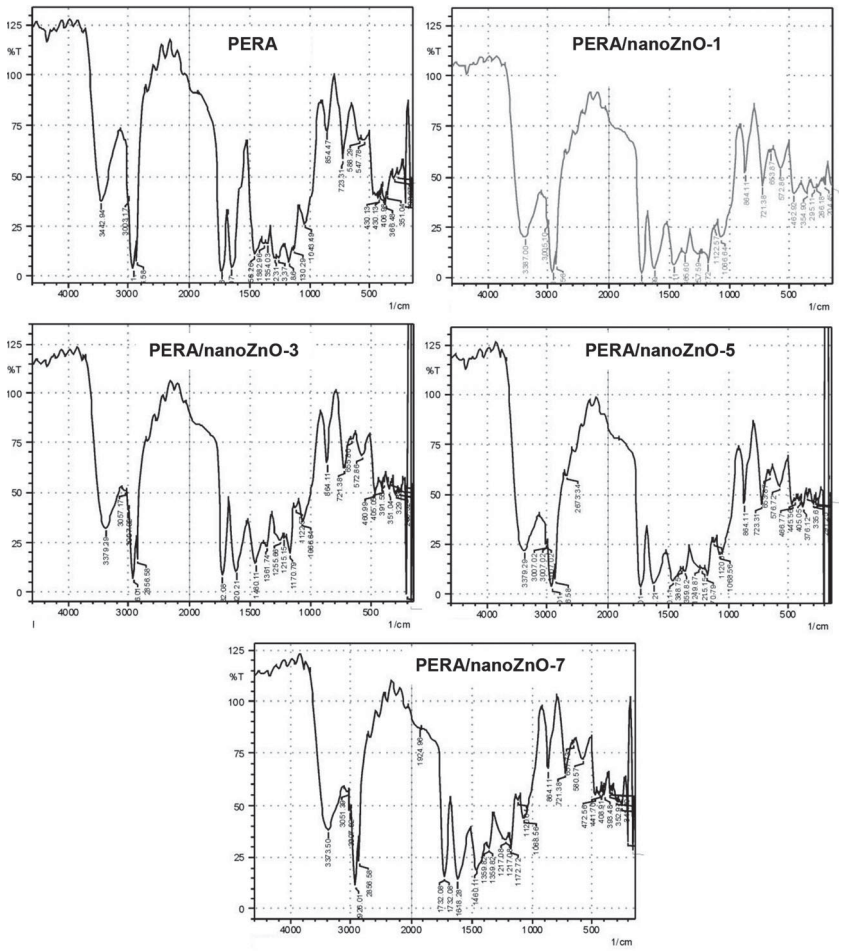


Figure 10.2 FTIR spectra of PERA and different compositions of PERA/nano-ZnO bionanocomposites.

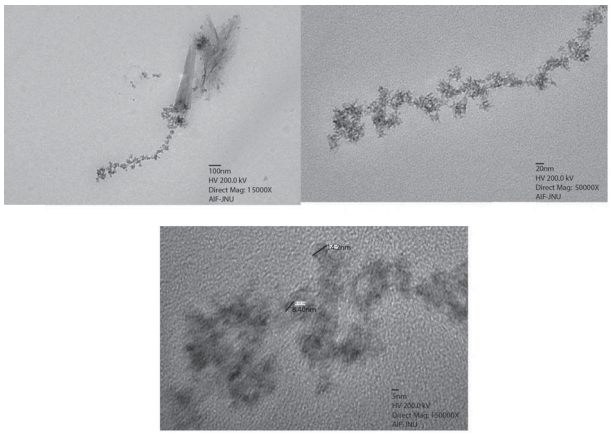


Figure 10.3 TEM images of PERA/nano ZnO-7 bionanocomposite at different magnifications.

10.3.2 Minimal Inhibitory Concentration

Table 10.2 summarizes the *in-vitro* susceptibilities of 09 *Candida* isolates of fluconazole-susceptible category. The data are reported as MIC required to inhibit 90% growth of the isolates. The results obtained showed that MIC varied with different isolates and was strain specific.

10.3.3 Disc Diffusion

The results summarized in Table 10.3 give the average zone of inhibition (mm), using standard discs of PERA, PERA/nano ZnO-1, PERA/nano ZnO-3, PERA/nano ZnO-5 and PERA/nano ZnO-7 (5 mg/ml) and fluconazole (100 µg/ml). All of the Candida isolates used in this study showed high degree of sensitivity as is evident from the zone of clearance in each case. Average zone of inhibition is greater (12.65 mm) for

Table 10.2 Minimum inhibitory concentrations (90%) of synthesized PERA and PERA/nano-ZnO bionanocomposites against different *Candida* isolates.

Bioactive Compounds	**Mean MIC_{90} (µg/ml)**
	Fluconazole- Sensitive (n = 09)
PERA	1500–2200
PERA/nanoZnO- 1	1300–1800
PERA/nanoZnO-3	1100–1300
PERA/nanoZnO-5	900–1000
PERA/nanoZnO-7	600–800

Table 10.3 Average inhibitory zone (mm) of PERA, PERA/nanoZnO-1, PERA/nanoZnO-3, PERA/nanoZnO-5 and PERA/nanoZnO-7 (5mg/ml) against *Candida* isolates as determined by disc diffusion assay.

***Candida* isolates**	**Average Zone of Inhibition (mm)**				
	PERA	**PERA/ NanoZnO- 1**	**PERA/ NanoZnO-3**	**PERA/ NanoZnO-5**	**PERA/ NanoZnO-7**
Invasive (n = 3)	1.51	4.33	5.13	8.03	10.23
Cutaneous (n = 3)	1.66	4.90	6.05	8.96	11.61
Respiratory (n = 4)	2.00	5.00	6.66	9.00	12.65

Fluconazole (100µg/ml) showed an average inhibitory zone (mm) of 15.3, 16 and 16.5 for invasive, cutaneous and respiratory isolates. n is number of isolates. Each isolate was tested in duplicate.

respiratory isolates when treated with PERA/nano ZnO-7 and least (1.51 mm) for invasive isolates when treated with PERA. Respective inhibition by fluconazole (100 μg/ml) was 15.3 mm, 16 mm and 16.5 mm for invasive, cutaneous and respiratory isolates. Results obtained in this study correlated well with MIC_{90} values obtained. The discs impregnated with DMSO (negative control) showed no zone of inhibition and hence 1% DMSO had no effect on the strains tested in the present study.

10.3.4 Growth Studies (Turbidometric Measurement)

Figure 10.4(a–e) shows the effect of different concentrations of PERA, PERA/nano ZnO-1, PERA/nano ZnO-3, PERA/nano ZnO-5 and PERA/nano ZnO-7 on growth pattern of *C. albicans* STD No 1305. Significant and pronounced effect is observed for other tested species (data not shown). Control cells showed a normal pattern of growth with lag phase of 4 hrs, active exponential phase of 8–10 hrs, before attaining stationary phase. Increase in concentration of test compounds leads to significant decrease in growth with suppressed and delayed exponential phases with respect to control. At MIC_{90} values, complete cessation of growth was observed for all the yeast species. No systematic difference was observed between isolates from various locations.

10.3.5 Proton Efflux Measurements

Proton-pumping ability of fungi mediated by H^+ ATPase at the expense of energy is crucial for regulation of internal pH and growth regulation of fungal cell. Fungal cells depleted of carbon source when exposed to glucose, rapidly acidify medium to generate proton motive force for nutrient uptake. *Candida* cells susceptible to the test compounds were examined for the ability to pump intracellular protons to the external medium (as

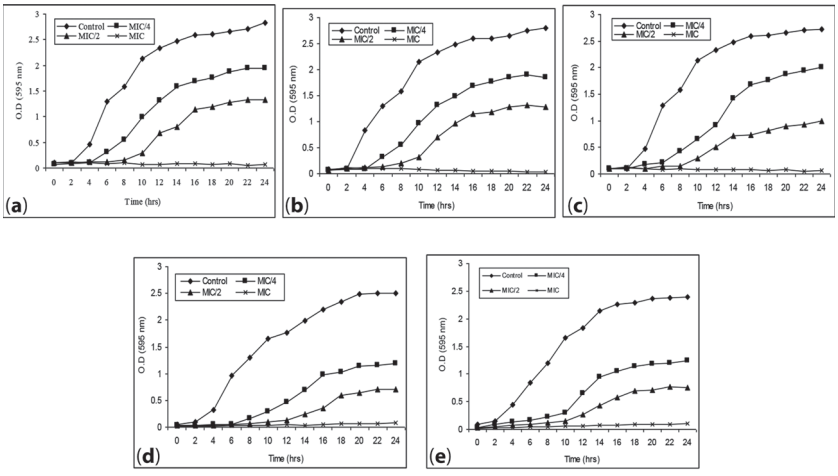


Figure 10.4 Effect of different concentrations of synthesized polymer and its composites against *Candida albicans* STD No 1305. Growth curve plotted against absorbance at 595 nm and time (hrs) shows complete inhibition of growth at MIC_{90} values. In presence of (a) PERA, (b) PERA/ nano ZnO-1, (c) PERA/ nano ZnO-3, (d) P ERA/ nano ZnO-5, (e) PERA/ nano ZnO-7.

Table 10.4 Effect of compound PERA, PERA/nanoZnO-1, PERA/nanoZnO-3, PERA/nanoZnO-5 and PERA/nanoZnO-7 on the rate of H^+-efflux by various *Candida* isolates at pH 7. Cells were suspended in 0.1 mM $CaCl_2$ and 0.1 M KCl at 25°C.

Incubation with	**Range of Relative H^+-Efflux ± S.D (% Inhibition)**		
	Invasive	**Cutaneous**	**Respiratory**
Control	1	1*	1**
PERA (MIC_{90})	0.82 ± 0.04 (17.36)	0.82 ± 0.05(16.89)	0.80 ± 0.01 (19.17)
PERA/nanoZnO-1 (MIC_{90})	0.74 ± 0.03 (25.07)	0.74 ± 0.05 (24.97)	0.73 ± 0.00 (25.52)
PERA/nanoZnO-3 (MIC_{90})	0.67 ± 0.06 (31.78)	0.67 ± 0.07 (32.01)	0.67 ± 0.02 (32.49)
PERA/nanoZnO-5 (MIC_{90})	0.59 ± 0.05 (40.37)	0.57 ± 0.14 (42.40)	0.57 ± 0.01 (41.99)
PERA/nanoZnO-7 (MIC_{90})	0.45 ± 0.05 (56.18)	0.44 ± 0.13 (55.03)	0.39 ± 0.02 (60.45)
Glucose (5mM)	3.42	3.71	4.01
Glucose+ PERA	3.14 ± 0.49 (8.36)	3.39 ± 0.86 (8.71)	3.65 ± 0.40 (9.01)
Glucose+ PERA/nanoZnO-1	2.99 ± 0.48 (12.68)	3.21 ± 0.81 (13.72)	3.40 ± 0.38 (15.03)
Glucose+ PERA/nanoZnO-3	2.72 ± 0.46 (20.68)	2.93 ± 0.79 (21.36)	3.14 ± 0.35 (21.69)
Glucose+ PERA/nanoZnO-5	2.51 ± 0.50 (27.03)	2.65 ± 0.99 (29.70)	2.88 ± 0.30 (28.02)
Glucose+ PERA/nanoZnO-7	2.14 ± 0.64 (38.35)	2.37 ± 0.92 (37.32)	2.26 ± 0.22 (43.36)

Control had an average (of 4 independent recordings) H^+-efflux rate of 5.63 n mol/min/mg cells in invasive isolates (1); 5.43 nmol/min/mg yeast cells in cutaneous isolates (1) and 5.33 nmol/min/mg yeast cells in respiratory isolates (1**). Values in parentheses give the %-age inhibition of H^+-efflux w.r.t. control.*

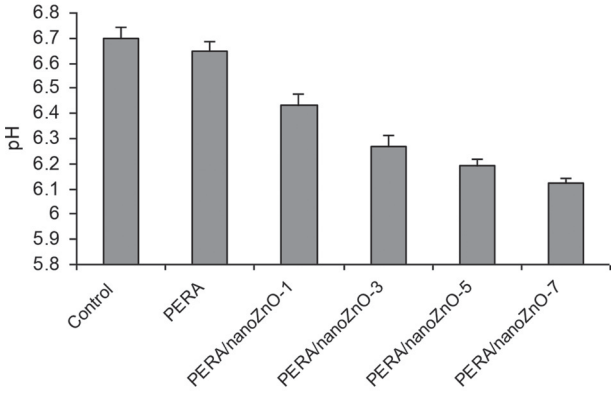


Figure 10.5 Intracellular pH in presence of test compounds in *Candida* sp. Mid-logarithmic cells were incubated with MIC_{90} of synthesized PERA and PERA/nano-ZnO bionanocomposites. Remarkable decline in pH as shown in figure is indicative of induced acidity.

measured by the alteration of pH of the external medium) in the presence of PERA, PERA/nano ZnO-1, PERA/nano ZnO-3, PERA/nano ZnO-5 and PERA/nano ZnO-7; Table 10.4 gives relative rates of H^+-efflux by *Candida* sp. in presence of test compounds (MIC_{90}). H^+-extrusion inhibition in invasive isolates was 17.36%, 25.07%, 31.78%, 40.37% and 56.18% when treated with PERA, PERA/nano ZnO-1, PERA/nano ZnO-3, PERA/nano ZnO-5 and PERA/nano ZnO-7, respectively. H^+-extrusion rate was also decreased to 16.89%, 24.97%, 32.01%, 42.40% and 55.03%, respectively for PERA, PERA/nano ZnO-1, PERA/nano ZnO-3, PERA/nano ZnO-5 and PERA/nano ZnO-7 in case of cutaneous isolates. In case of respiratory isolates the decrease was 19.17%, 25.52%, 32.49%, 41.49% and 60.45% when cells were treated with PERA, PERA/nano ZnO-1, PERA/nano ZnO-3, PERA/nano ZnO-5 and PERA/nano ZnO-7, respectively. Glucose (5 mM) stimulated H^+-efflux in all the strains by 4–5 folds. Glucose stimulated H^+-efflux was also inhibited by test compounds (Table 10.4). Detailed studies of test compounds on the same can give us more insight into the possible mechanisms of action.

10.3.6 Measurement of Intracellular pH

The internal pH of yeast cells is maintained between 6.0 and 7.5 by plasma membrane ATPase activity. We tried to investigate whether cells with normal plasma membrane ATPase activity maintain the constant internal pH as compared to cells with low plasma membrane ATPase activity. Figure 10.5 shows changes in the pattern of pHi with control and treated cells. Only yeast control cells with normal plasma membrane ATPase activity maintain pHi (6.52), while the treated cells show increase in internal acidification. The decrease in pHi was more in cells exposed to PERA/nano ZnO-7 than rest of the synthesized composites.

10.4 Discussion

Emergence of multidrug resistance in human and animal pathogenic fungi, as well as undesirable side effects of antibiotics, has triggered immense interest in searching for

new antimicrobial agents. The synthesis of the new bionanocomposite (PERA/nano ZnO) via microwave-assisted methods (green method) by utilization of bio-derived resource for antifungal agent is a small step towards this. PERA/nano-ZnO bionanocomposite was synthesized *in situ* between the reaction of MicHERA, zinc acetate (dehydrated) and maleic anhydride below 100°C under microwave irradiations without any volatile organic solvent. PERA/nano-ZnO bionanocomposite was synthesized in 6 minutes, a very short time, which is a characteristic of microwave-assisted synthesis (dielectric heating) [67]. In the present microwave-assisted preparation, dielectric properties of reactive constituents are collectively responsible for the synthesis of PERA/nano ZnO at lower temperature and time [67]. The overall synthesis protocol is simple, clean and completed in a very short time period as compared to the other previously reported complex methods of nanocomposites preparation [68]. The synthesized bionanocomposites showed photoresponse when exposed under UV light, that is, they changed from light yellow color liquid to green color liquid of bionanocomposite (Figure 10.1). The photoresponse of bionanocomposite is correlated to wide band energy gap of *in-situ* synthesized nano ZnO within PERA matrix, since ZnO is characterized by its electron mobility, wide band gap (3.37 eV) and large exciton binding energy (60 meV) [69].

In FTIR spectral studies, the characteristic peaks of PERA and nano ZnO [69,70] both presented in PERA/nano-ZnO spectra are correlated to the formation of PERA/nano-ZnO bionanocomposites. The intensity of the stretching frequency of Zn-O increases with metal contents. The close examination of spectra reveals shifting and broadening of amide linkages (>C=O amide) in PERA/nano-ZnO spectrum as compared to PERA spectrum, which correlated to hydrogen bonding interaction between >C=O amide and PERA with the surface of nano ZnO [42].

Fungal infections are caused by eukaryotic organisms and for that reason they generally present more difficult therapeutic problems than do bacterial infections. The fungal cell wall may be considered to be a prime target for selectively toxic antifungal agents because of its chitin structure, absent from human cells [71]. Current drug treatments are effective, but resistant strains and intrinsically resistant species are emerging fast. Moreover, the treatment cost, associated host cytoxicity and the fact that most available antifungal drugs have only fungi-static activity justify the search for new strategies. We demonstrated that the synthesized compounds at MIC_{90} values exhibit fungicidal and not fungistatic activity, by halo assay and growth curve studies against all the *Candida* isolates. *In-vitro* studies have shown that PERA, PERA/nano ZnO-1, PERA/nano ZnO-3, PERA/nano ZnO-5 and PERA/nano ZnO-7 significantly inhibit the growth of fluconazole-susceptible *Candida* isolates. Bionanocomposites showed improved antifungal performance over the virgin polymer [PERA], attributed mainly to the presence of ZnO nanoparticles. Synthesized bionanocomposites exhibited higher antifungal activity (lower MIC – Table 10.2; higher zone of inhibition – Table 10.3) as the percentage of ZnO precursor varied. These results gave a good indication of the overall antimicrobial effectiveness of each synthesized compound with higher content of ZnO. As discussed previously, micron-sized ZnO particles could not counteract fungal growth [45]. The synthesized bionanocomposites are highly efficient to counteract the yeast physiology by the biocidal action of nano-sized ZnO particles attributed to their large surface-to-volume ratio [69,72]. Figure 10.4 reveals that the control cells show normal growth pattern in liquid medium.

At lower concentration of the synthesized compounds, a slight decline in curve was observed as compared to the control, whereas at MIC_{90} values of the test agents, the S-shaped curve declined to a flat line, showing almost complete arrest of cell growth. Anticandidal activity order on solid medium lead to a similar conclusion; the yeasts were found more sensitive towards PERA/nano ZnO-7 followed by PERA/nano ZnO-5, PERA/nano ZnO-3, PERA/nano ZnO-1 and PERA. The conclusion that compound PERA/nano ZnO-7, which contains higher percentage of nano ZnO precursor, has higher antifungal activity than rest of the systems is based on the differences in compound concentrations needed to inhibit yeast growth (Table 10.2).

Candida isolates showing susceptibility to the test bionanocomposites also showed inhibition of H^+-ATPase-mediated proton pumping, suggesting that the two events are linked. It is to be noted that the inhibition of H^+-ATPase function was achieved at MIC_{90} concentrations of compounds. The decrease in H^+ extrusion being less when cells were exposed to the test compounds in presence of glucose. It is well established that plasma membrane ATPase undergoes modification in glucose medium [73]. Glucose-induced acidification of the external medium by yeast cells is a convenient measure of H^+-ATPase-mediated proton pumping [74]. The enzyme may be existing in a different conformational state in the two situations. It is thus possible that the test compounds may be directly interacting with the enzyme, which serves as the primary reason for their antifungal activity. Regulation of pHi appears to be a fundamental prerequisite for growth of *Candida* and activation of plasma membrane ATPase, as it is involved in maintenance of pHi [75]. We therefore studied the role of plasma membrane ATPase activation in the regulation of pHi, in control as well as treated cells. The pHi was near neutrality in absence of test compounds and then declined to 6.65 (PERA), 6.43 (PERA/nano ZnO-1), 6.27 (PERA/nano ZnO-3), 6.19 (PERA/nano ZnO-5) and 6.12 (PERA/nano ZnO-7) at concentrations inhibitory to H^+-efflux and growth of *Candida*.

10.5 Conclusion

The microwave-assisted synthesis via *in-situ* approach has resulted in both nano ZnO and poly(ester-ricinoleamide), leaving the nano ZnO dispersed in poly(ester-ricinoleamide) as a bionanocomposite. The antifungal study shows that the synthesized composites have markedly inhibited growth of different *Candida* isolates. The major site of action of the test compounds appears to be the inhibition of H^+-ATPase-mediated proton pumping activity. The reason to report on these data is that these synthesized compounds might reach clinical importance in near future, defining a new class of antimycotics. The novelty of this study may reveal a promising antifungal agent to cure fungal infections. The outcome of this research work may provide a basic platform for further research in(green) pharmaceutical applications (topical or transdermal) for the treatment of fungal infections and in wound care. Studies on biodegradation behavior of these bionanocomposites are still in progress in our laboratory. PERA/nano-ZnO bionanocomposite may find promising applications as novel bio-derived antimicrobial and biodegradable materials for different biomedical fields.

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11

Improving Matters of the Heart: The Use of Select Pharmaceutical Polymers in Cardiovascular Intervention

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Abstract

Both synthetic and natural polymers, and sometimes a combination of the two, are used in the design and manufacture of multiple devices and implements intended for use in the field of cardiovascular intervention. This chapter provides an overview of some of these polymeric materials with respect to the following five broad domains in cardiovascular science: 1) drug-eluting stents, 2) prosthetic devices, 3) gene therapy, 4) tissue engineering, and 5) injectable biopolymers. Within each section a detailed introduction covers the historical perspective on the need for the emergence of a specific polymer, its advantages and limitations of use and in most instances, its physicochemical characteristics, before delving deeply into the particular cardiovascular device the polymer is used in. It is the author's primary intention to provide a concise synopsis of the broad and multivariate aspects relating to polymeric materials currently in use for cardiology.

Keywords: Gene therapy, tissue reconstruction, injectable biopolymers, polymeric drug-eluting stents, cardiovascular polymers

11.1 Pharmaceutical Polymers Used for Drug-Eluting Stents

11.1.1 Introduction and Historical Perspective

Till the early 1990s, there was a lack of interventional procedures, except for ballooning angioplasty, to correct artery occlusion. In fact, options to repair collapsed arteriolar walls were limited. One challenge was accessing the damaged vessel wall without extensive surgery in order to repair it. The coronary arteries which supply blood to the heart muscle have a lower diameter than most other vessels in the body making them more prone to occlusion. If a coronary artery remains occluded over a long period of time, the region of the heart that it is supplying blood to will infarct. Further, whether the occlusion is due to a fibrin clot, or lipid deposits on the inner side of the walls of

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the arteries, or due to other underlying disease such as atherosclerosis, occlusion may lead to a breach of the vessel wall. Damaged and ruptured coronary arteries need to be repaired and compromised vessel walls need to be reinforced. Thus, as a means of addressing this challenge, the 1990s saw the advent of stents, which were initially made of metals, and were used to reinforce collapsed and damaged vessel walls.

Although stenting as a technique had initial success when it was introduced, by the middle of the 90s, it was fast losing clinical support since stents were generally associated with the problem of extensive thrombosis *in situ* in patients. Another issue was that of restenosis, wherein the innermost lining of the arteriolar wall, known as the intima, would grow back due to mitosis of the intimal cells over time, resulting in yet another occlusion, this time due to the stent itself. To overcome these new challenges, the design and fabrication of Drug-Eluting Stents (DES) was envisioned and subsequently achieved. Drug-eluting stents were reported to be very successful in eliminating adverse patient events and in the decade since have emerged as an extremely useful fix to the problem of restenosis and in-stent thrombosis.

DES contains an outer metal body, generally in the form of a screw but may be shaped otherwise, and a polymer base in the center. To the polymer base is sequestered a drug of choice that upon local release prevents intimal regrowth and consequently arteriolar restenosis. Of course, any drug system such as anticoagulants may be bonded, adsorbed, or imbibed into the polymer-base in the DES for eliciting the desired physiological response. Thus, with the timely development of DES, the challenge of restenosis was met since most DES served more than simply a structural role; they simultaneously functioned as reservoirs, resulting in a controlled and prolonged release of the drug, particularly at local sites. However, despite these advances, one problem still plagued DES usage in clinical practice: the lack of tissue integration of the stent with the vascular tissue, and the development of immune rejection and chronic inflammation.

Further advances in technology offered the solution of surface modification of the metal structure of the DES by chemical or physical adsorption of biopolymers or synthetic polymers that would allow enhanced cell adhesion following placement of the stent. Thus, pharmaceutical polymers may be used not only in the design of the actual stent, but also to coat stent surfaces to augment tissue compatibility. At the present time there are a few DES which are approved by the Food and Drug Authority (FDA) for use in humans. These are further discussed below.

11.1.2 Polymers Used in Drug-Eluting Stents

11.1.2.1 Polyethylene-co-vinyl Acetate (PEVA) and Poly n-butyl Methacrylate (PBMA)

Cypher® is a first-generation DES that releases the immunosuppressive agent sirolimus (brand name Rapamune) which is bound to polymers embedded in this stent. The Cypher system has a stainless steel base which is cut into a sinusoid shape and is coated with a blend of two polymers. The blended polymers are polyethylene-co-vinyl acetate (PEVA) and poly n-butyl methacrylate (PBMA). A combination of the two polymers mixed with sirolimus (67%/33%) makes up the basecoat formulation. Another coat of

the PBMA polymer is applied to the surface of the DES, which helps in making this a controlled release formulation. The use of PEVA for this purpose originated with the design of controlled release formulations being implanted into patients, since ethylene-co-vinyl acetate can be mixed with an organic solvent to which the drug of choice has been pre-added, and this mixture can be frozen in blocks at −80 degree Celsius. These blocks could subsequently be used as controlled release formulations. Thus, the use of this polymer in the Cypher system assists not only in the construction of a mechanical repair device, but also in the creation of a formulation to control the release of drugs, preventing intimal regrowth and restenosis.

First-in-man clinical trials with the Cypher stent system were performed at the Instituto Dante Pazzanese de Cardiologia in the year 2000, comparing a moderate-release cypher stent with a slow-release one in 15 human patients. At the time stent-technology had been an emerging field and this clinical trial helped establish the therapeutic benefits of DES use by demonstrating slowed neointimal growth in stented patients [1].

11.1.3 Polymers Used for Paclitaxel Stents

Paclitaxel is an impressive anticancer and immunosuppressive drug , which works by inhibiting cellular microtubule polymerization. It can be used to prevent the intimal regrowth and in-stent restenosis often associated with vascular stents. Based on these properties of paclitaxel, over time a number of polymers have been used in different stent systems. Some of the more commonly used polymers, along with the corresponding DES are detailed below.

11.1.3.1 Poly(lactide-co-Σ-caprolactone)

Poly(lactide-co-Σ-caprolactone) is a derivative of polycaprolactone, which is a biodegradable polyester used in the synthesis of polyurethane polymers. Drachman *et al.* placed stents containing the polymer poly(lactide-co-Σ-caprolactone) into pig vascular beds to study the effects of the stent on neointimal regrowth. They reported that the paclitaxel releasing stent was able to curtail vascular intimal regrowth and in-stent restenosis at all the time points they tested from 7 to 180 days [2]. However, in studies conducted in other animals it became apparent that arteries did not heal completely following stent-mediated release of paclitaxel [3].

11.1.3.2 Polylactic Acid Polymer

Polylactic acid is a biodegradable polymer of lactic acid that can be derived both from compounds such as starch, or chemically synthesized in the laboratory. It is a very flexible polymer and has been used for manufacturing a variety of different objects. In the field of interventional cardiology, this polymer is used within a DES to bind the immunosuppressive drug everolimus, which is similar to sirolimus discussed above. The S-Stent from the company Biosensor contains everoliums bound to polylactic acid. Two versions of the stent were tested in pigs for restenosis [4], low and high dose everolimus-releasing stents, and both were found to be nearly equally effective in preventing restenosis.

11.1.3.3 Poly n-butyl Methacrylate

Poly n-butyl methacrylate (PBMA) is a polymer used in the second-generation drug-eluting stent called Xience V®, which is an everolimus-eluting stent which has been in use in Europe for a few years. This cobalt-based stent uses a double polymer system comprised of PBMA and that adheres to the stent and the drug coating polymer, polyvinylidene fluoride, which is made up of vinylidene fluoride and hexafluoropropylene monomers (PVDF-HFP). PBMA is a homopolymer with a molecular weight in the range 264 to 376 kDa. PVDF-HFP is a non-erodible semicrystalline random copolymer with a molecular weight range of 254 to 293 kDa.

This product is different from the others described above because not only does it employ a different polymer blend, but it also uses a double polymer approach, where one polymer is used as a primer and the other as a drug reservoir. In this device, PBMA is used to enhance adherence and PVDF-HFP used to enhance drug binding. Additionally, no top coat is applied to the device. The polymer blend and the everolimus drug are mixed in the ratio of 83%/17% w/w polymer to drug, and subsequently this mixture is added to the PBMA coated stent.

This dual use of polymers and a double-layered engineering strategy affects drug release parameters. The Xience V stent has been reported to release 25% of its total everolimus content on the first day after placement in the vascular bed, with the remaining 75% of drug released over 4 months, while most of the drug release remains in the local vascular microenvironment with minimal systemic release of the drug [5].

11.1.3.4 Phosphorylcholine

Phosphorylcholine is one of the head groups of phospholipids. Its polymer is used as a coating for the Endeavor drug-eluting stent system. This DES finds popular use in the clinical setting in European countries. A second generation DES, it is composed of a cobalt driver platform and a phosphorylcholine polymer coating which binds and releases the immunosuppressive drug zotarolimus in a controlled release form. This device has been evaluated in clinical trials, known as the ENDEVAOR I and II trials published in 2005 [6]. In later and more extensive clinical trials in patients, one problem observed with this DES was in-stent thrombosis [7], which in the ENDEVAOR IV trial was addressed by a comparison between the Endevaor and Cypher systems in 1,500 patients, and was reported to evince the same number of vascular failures between the two DES [8].

11.2 Pharmaceutical Polymers Used in Cardiovascular Prostheses

11.2.1 Introduction and Historical Perspective

Early Materials: 1800s – mid-1970s

The use of external media in the cardiovascular system historically followed the surgical need for the repair or grafting of vascular structures. Surgical intervention for correcting anatomical defects, or repairing torn blood vessels necessitated the hunt for materials

that could be employed to replace damaged tissue. Interestingly, since the early 1800s, a number of materials are known to have been tried in patients before the use of polymers about a hundred years later. Early materials varied from substances like glass to metals, but such repairs usually broke, or underwent thrombotic build-up, or were overcome by anastomosing blood vessels that ultimately obviated the repaired vessels for blood flow. Next in vogue were textiles made from a variety of materials. The use of textiles in cardiovascular surgery followed the demonstration that a layer of textile could activate fibrin, causing this protein to stop the bleeding, and therefore facilitate wound repair of the ruptured vessels. Soon, disparate materials were being tried, including polyvinyl alcohol, polyamide, polyacrylonitrile, polyethylene terephthalate, polyurethane, and polytetrafluorethylene—any material from which fibers could be extruded, which could be fabricated into the desired shape and form. Of these, since the mid-1970s, polyurethane and polytetrafluorethylene (PTFE) were the most successful and were used in a variety of ways in cardiovascular applications. PTFE is discussed in detail below. Although PTFE textile fibers are no longer in use for vascular grafts due to handling issues, it continues to be used in the construction of other cardiovascular prosthetic devices.

1980s and the Use of Silicone

The next interesting usage of a polymeric material for the construction of cardiac prostheses was the employment of silicone, which in the form of a silicone ball in a cage-like structure was used as a prosthetic substitute for a heart valve. The silicone material showed great resilience since after multiple years in the patient, the ball did not show signs of wear. One reason for this remarkable success was the lack of adherence of cells to silicone.

1990s and the Use of Biopolymers

The past two decades have again seen a significant transition from synthetic to biopolymer use in the manufacture of implements and in the graft process itself. Biopolymers are in much use not only as construction materials, but also for the purpose of surface modifications of prosthetic devices. Biopolymer-based surface modification is usually achieved by anchoring a biopolymer by either chemical or physical means via a conjugation-based chemical reaction or adsorption, respectively, to the surface of the prosthetic device. The primary purpose of such procedures is to enhance tissue integration of the implant by promoting cell adherence and ameliorating immunogenic rejection responses. Thus, a variety of biopolymers, usually proteins, but also polysaccharides, or smaller peptide sequences have been employed as surface coats. For instance, the protein collagen, which is a connective tissue protein, has a tripartite fibrillar structure, and is the most abundant protein in the human body, is extensively employed as a surface modifier for a variety of prosthetic devices and implants, as well as to coat the surfaces of vascular grafts. Collagen coating enhances early recruitment of cells to the graft, promotes cellular adherence and improves tissue integration. Additionally, collagen can itself be further chemically or physically modified to allow further anchorage of specific proteins or other molecules. An area where this property is useful is the construction of nanoparticles, as discussed below.

The Past Two Decades – Biopolymers and Tissue-Engineered Cardiovascular Prostheses

The latest wave in the field of cardiovascular prosthetic devices is the construction of artificial tissue grafts, whereby cardiocytes and other cells are grown on engineered

platforms and these are subsequently grafted into the organ. While largely still an experimental technique, multiple groups have investigated the feasibility of this approach in small animal models such as rodents in the lab. In some specific instances, clinical trials are also underway to test the viability, adverse effects, and half-life of similarly constructed platforms. With respect to this latest development, pharmaceuticals as well as biopolymers are now being employed in two novel ways: either for the construction of tissue-engineered scaffolds, or the surface modification of such platforms to enhance tissue integration and revascularization following implantation. The first step in the manufacture of such tissue-engineered prosthetics is the construction of the scaffold, which is then seeded with the specific cells. Scaffolds are generally created by the process of self-assembly of nanoparticles formed from synthetic polymers or biopolymers. For instance, collagen, gelatin (a heat produced breakdown product of collagen), or the protein albumin may be used as biopolymers for the construction of nanoparticles, which are subsequently assembled into scaffolds. Similarly, instead of proteins, nanoparticles may be formed from other biopolymers such as the polysaccharides alginate and chitosan, or from a variety of synthetic pharmaceutical polymers.

Selection of the specific polymer that is eventually used for the construction of the particular cardiovascular implant or prosthetic device is influenced by a number of criteria, which are discussed next.

11.2.2 Factors Affecting Selection of Polymer

The choice of the specific polymer used for the construction of vascular grafts depends on a multitude of factors, particularly vessel characteristics such as the diameter of the blood vessel under consideration, the place in the body where the blood vessel is located, the caliber of the prosthetic device required, among others. In general, the long-term success and value from a vascular graft may be seen in large caliber arteries, which have a diameter greater than 8 mm. This is largely due to the establishment of massive blood flow in large caliber arteries which prevents thrombosis. It is important to note that the material used most commonly for the construction of blood vessel grafts is the patient's own large vessel, usually aorta-illiac substitutes since their graft longevity is reported to be as high as 90% [10].

11.2.3 Specific Polymers Used in Cardiovascular Applications

11.2.3.1 Polyethylene Terephthalate (PET)

Polyethylene terephthalate (PET) is a polymer that has been extensively used in cardiovascular implements and patches for multiple decades, ever since its introduction in England in 1939, followed by the development of its fiber form by DuPont in 1950. Sold in the United States under the brand name Dacron, PET is a thermoplastic polyester polymer resin used in the textile industry in the design of synthetic fabrics [9]. PET is composed of alternating chains of glycol and terephthalic acid,

with a molecular formula -O-C=O-C_6H_4-O-C=O-CH_2-CH_2-. It is a highly flexible material that lends itself to multiple and diverse uses as far ranging as the manufacture of water bottles to the synthesis of cardiovascular patches for vascular grafts and shunts. Its tensile strength ranges from 59–72 MPa, with an elongation percentage from 50–300%.

One reason for the success of Dacron for use in the construction of vascular prosthetic devices stems is the ease with which patches of varying porosities can be constructed by the employment of slight variations in manufacturing technology. There are two main forms of the manufactured Dacron fabric, one based on knitting, while the other is obtained by weaving. Both forms differ markedly in their porosity, with the knitted patches used for grafts having an appreciably higher porosity than the woven ones. The porosity of a Dacron fabric refers to its water permeability. Interestingly, Dacron fibers can also be crosslinked by low concentrations of formaldehyde or glutaraldehyde, allowing for the manufacture of a number of cardiovascular prosthetic devices. However, these manufactured Dacron materials may be coated with collagen, albumin or gelatin. It is relevant to know that the albumin coating may degrade 2–8 weeks post grafting, while uncoated Dacron has a higher stability profile and can last up to 10 years after implantation.

11.2.3.2 Expanded Poly(tetrafluoroethylene) (ePTFE), Teflon, or GoreTex

Although first introduced by DuPont in 1936, PTFE was not patented till the 1960s when it was first introduced into the medical market. Interestingly, it is a compound formed wholly of carbon and fluorine. Better known by its brand name, Teflon, like PET discussed above, it is vastly flexible in the uses to which it can be adapted. It is a high molecular weight solid, the fibers of which have a tensile strength in the range of 14–35 MPa and an elongation stretch percentage of 200–400 percent. In the context of cardiovascular applications it is used in the manufacture of catheter coating, oxygenator membrane and vascular grafts. Interestingly, the modern form of PTFE, known as expanded PTFE or ePTFE, which is actually used in the design of vascular grafts, was patented in 1996, and was a microporous material.

There are a number of advantages that ePTFE offers for use in the manufacture of cardiovascular graft material, prime among which is its inertness, which increases its safety profile, making it ideal for use in biological systems. Furthermore, ePTFE has an electronegative surface which prevents its interaction with most blood components. The main difference between ePTFE and PET lies in the manufacture process used for ePTFE, which is made by an extrusion process in which the main material is in the form of a "node," from which tube-like long processes are extracted. Thus, there is a fundamental difference in the architecture of Dacron and Teflon fibers. The fiber length of ePTFE is usually measured as the length of the fiber in between two nodes, known as internodal density, which for ePTFE is between 30 to 90 μm. Although ePTFE has been used for cardiovascular device manufacture for a few decades, in a clinical trial in reported in 1992, Kohler recorded vascular ingrowth on a highly porous ePTFE

vascular bed, and it was noted that the regrowth did not extend more than halfway into the graft [11].

11.2.3.3 Disadvantages of PET and ePTFE

Although both PET and ePTFE are generally inert, biologically resistant, tough and lasting polymers with vastly reduced biological interactions, they do have some disadvantages. Prime among these is vascular regrowth. There is a predictable sequence of events that precede revascularization beginning with fibrin activation and deposition which occurs directly onto the graft surface. In 1993, Guidoin *et al.* demonstrated that this stage was followed by collagen deposition accompanied with minimal cell invasion [12]. Later studies showed similar processes occurring with both Dacron and Teflon fibers and thus no difference between the two, with neither one offering any advantage over the other [13].

11.2.3.4 Polyurethane

Polyurethane is a carbamate [-NH-(CO)-O-] containing polymer that is composed of three monomeric units. Different permutations and combinations of constituent monomers lead to differences in characteristics, flexibility, and ultimately use. Of the three monomeric units, one is a derivative of diisocynate, the second unit is usually a polyol, and the final third monomer is a chain extender used for lengthening the polyurethane. Each monomer contributes specific physical and chemical characteristics to the polyurethane matrix. Diisocynate lends mechanical strength and hardiness, while the polyol makes the material malleable and flexible. Pellethane, which is poly(ether) urethane, has a tensile strength ranging from 35 to 48 MPa and an elongation percentage of 350–600 percent. It is used for the manufacture of intraaortic balloons, catheters, tubing and percutaneous leads.

There have been three generations of polyurethane products, at least from the perspective of their medical usage, particularly in the cardiovascular field. Available since the 1960s for the manufacture of medical implements and devices, the first polyurethanes were not biologically stable since they were prone to catalytic hydrolysis in the body [9]. Modifications were made to bolster the polyurethane, resulting in the creation of a copolymer that resisted catalytic degradation [14]. However, this also had the unintended consequence of making the polymer more prone to oxidative damage. This form of the polymer was approved by the FDA for hemodialysis grafts in 2000 [15]. Some examples of these generational polymeric differences are polyester polyurethane, polyether polyurethane and finally the polycarbonate polyurethane, which was both oxidative and hydrolytically stable. In fact the latest generation of polycarbonate polyurethane made vascular grafts was in phase I clinical trials in 2003. This was a direct reflection of their superior performance compared with Dacron or ePTFE fibers in experimental animal models. In a study that followed canine vascular grafts for a period of 36 months, histological examination following the study showed that the grafts had little intimal growth, and still maintained excellent compliance [16].

11.3 Pharmaceutical Polymers Used for Gene Therapy

11.3.1 Introduction to Cardiovascular Gene Therapy

Another use of pharmaceutical polymers is in the field of gene therapy. Gene therapy is a modern approach to ameliorating pathology arising out of deficiency of a normal or expression of an aberrant gene in a specific tissue. With the advent of material sciences, the discovery of new polymers and advancement in both technology, as well as the molecular basis of cardiovascular disease, gene therapy, until recently regarded mostly as a futuristic technology, has offered new hope to patients suffering from cardiovascular complications. Interestingly, the vast number of recently concluded and on-going clinical trials of this novel therapeutic approach attest to the growing need and popularity of this technology. Until 2010, 1347 clinical trials based in gene therapeutics were in effect worldwide, with 11 clinical trials conducted solely for gene therapy for myocardial events [17]. A number of adverse effects were related to the delivery of genes to patient tissue in the clinical setting.

The cardinal premise of gene therapy is the delivery of selected genes to living tissue in patients using specially engineered vectors that exhibit the ability to fuse with cell membranes and deliver their genetic cargo to the intended target organ or tissue. The idea emerged from experimental molecular biology, specifically with the discovery that special viruses may be engineered such that their core genetic material could be replaced with genes intended for therapy, while the outer lipoprotein shell of the virus was maintained intact. This led to the possibility of efficient delivery of genes via a mechanism known as "transduction." Since the original viral genes were largely replaced with therapeutic genes, it was thought that the incidence of adverse events would be negligible. The technology was greatly favored, at least in theory, since it offered a medium of efficient delivery of genes to living tissue, which posed complex biological and therapeutic challenges of temporal and spatial specificity. To elaborate on the latter, consider the case of myocardial ischemia. Since dead tissue infarcts may be spatially dispersed among living tissue patches in the patient heart, gene therapy would only be specifically desirable for the necrotic and damaged tissue. This scenario may be further complicated in advanced patients, in whom the distribution of necrotic tissue changes with time.

11.3.2 Cardiovascular Gene Delivery Systems

In general, cardiovascular gene therapy may be achieved by the use of any one of the following materials for encapsulating DNA. The methods used may be classified into viral and non-viral techniques. The more common, as discussed above, are viral vectors, which may be further subclassified into 1) retroviral, 2) adenoviral, or 3) receptor-targeting modified adenoviral, and, 4) lentiviral vectors. Viral vectors offer the added advantage of targeted delivery, especially useful in this context, since the viral lipoprotein shell may be engineered to express novel proteins that allow binding affinity to receptors expressed by the compromised tissue, thus allowing targeted delivery of genes only to

tissue that is damaged. However, in spite of this, severe complications and even death has been reported in the past with use of viral vectors for gene delivery in human patients. Although arguably, these occurred more than a decade and half ago, and since that time technology has vastly improved. Viral vectors will not be discussed in this chapter. Non-viral methodology for gene therapy can be achieved by either chemical or physical methods. While chemical methods are mentioned below, the physical methods include magnetofection, electroporation, the use of gene guns, and particle bombardment.

An interesting and quite modern use of polymers in cardiovascular gene therapy is the ultrasound driven microbubble-assisted delivery of genes to target organs, and this technique, along with the polymeric materials used therein, shall be discussed separately as a distinct category.

11.3.3 Ideal Polymeric Characteristics for Use in Gene Therapy

To overcome potential and serious side effects, polymers are being researched as delivery vehicles for genetic material, instead of engineered viral vectors. A number of non-viral vectors emerged in the 1990s as putative vehicles of genetic material. Prime among these were 1) cationic liposomes, 2) HVJ-liposome conjugates, 3) nanoparticles, and 4) polymers. However, the intended use of the polymers in patients, limits the usable ones to those that exhibit the following selection criteria. To be used in the manufacture and delivery of genetic material in humans the selected polymers must be 1) non-cytotoxic, 2) should exhibit ease of fusing with the cell membrane, 3) should be resistant to endosomal lysis, and 4) should protect the enclosed DNA. Additionally, from an engineering perspective, they should be easy to work with and to use for manufacture, particularly ensuring homogenous encapsulation of DNA to optimize delivery.

A number of other salient features of polymers, especially when compared with their viral counterparts for use as gene vectors, make them highly desirable, particularly from an engineering point of view. Most polymers are 1) nonexpensive, 2) lend themselves to easy and flexible quality control procedures, 3) have extended safety profiles, the latter particularly significant when considering the relative inertness of DNA-encapsulating polymeric vectors that evince limited binding to unintended tissue, and 4) do not possess a ceiling for amount of DNA packaged into them. In contrast, viral vectors only permit lower DNA volumes.

11.3.4 Polymers Used in the Design of Cardiovascular Vectors

In general, the more successful polymers used in cardiovascular gene therapy are derivatives of polyethylenimine. There is also a lower molecular weight, water soluble version of this polymer. Recently, modified forms of this polymer have also become available such as the poly(amido polyethyleneimines).

11.3.5 Ultrasound-Targeted Microbubble Destruction (UTMD) for Cardiovascular Gene Therapy

Microbubbles are small-scale vectors that can carry drugs and genetic cargo into interstitial spaces (microscopic spaces between cells within an organ), due primarily to their

small size and the elasticity of their wall architecture, which allows them to "squeeze" between anatomical structures. Traditionally, microbubbles were used as vectors for enhanced contrast agents for diagnostic techniques such as ultrasound and contrast-enhanced Magnetic Resonance Imaging. However, recently this technique has been successfully adapted for the delivery of genetic material in a more targeted manner, particularly to the cardiovascular system.

UTMD works in the following way. Microbubbles incorporating DNA are injected into the bloodstream and monitored till they reach the intended end organ, for instance the heart. Subsequently, once they extravasate, which entails exiting the blood vessels and "trapping" in the interstitial space, they are exposed to ultrasound waves, resulting in microbubble oscillation and eventually at high frequencies, breakage, with the slew of encompassed DNA-vectors getting pooled locally. Additionally, the application of ultrasound to the micobubbles, which results in oscillations, also causes the development of local shear forces, a phenomenon known as "microstreaming." Microstreaming results in the creation of pores in the local cell membranes, which allows transduction of genes into the vascular cells.

11.4 Pharmaceutical Polymers Used in Tissue Engineering

The latest advancement in technology is the construction of synthetic platforms using a variety of base materials that would allow cell attachment and growth on the platform itself. This would enable the construction of a "scaffold" which could then be used in place of a synthetic graft, extraneous object, or a transplanted organ from another individual or species. For instance, cardiocytes can be grown on such platforms and once adequately populated, the entire reconstructed synthetic tissue can be implanted into the damaged organ in humans. Such an approach that leads to the creation of artificial organs or replacements for damaged organs is known as tissue engineering.

The main advantage of engineered tissue is a lack of immune response since the scaffold is populated with cells derived from those originally obtained from the patient. Because of this, the cells populating the scaffold are compatible with tissue antigens. Further, use of scaffolds to engineer tissues helps solve a complex biological problem: that of spatial and temporal molecular cues that help maintain tissue integrity. Since tissue are not merely composites of cells, but contain a variety of cells embedded in a "live" ground-substance or matrix, it is important to consider the structure, composition, synthesis and maintenance of the matrix of the tissue, the co-called extracellular matrix (ECM). In most tissues, for example, cells adhere to this matrix using special adherence proteins. In fact the matrix is generally composed of collagen, and depending on the organ may also contain elastin, fibronectin, and other proteins. These ECM proteins significantly offer not only an anchoring platform to the cells of the tissue, but also temporo-spatial cues for processes such as tissue growth, repair and regeneration. Thus, engineered scaffolding based artificial tissue constructs provide an effective substitute for replacing parts of a damaged organ and in the future may actually offer fully replaceable organs.

Interestingly, tissue engineering is not an esoteric concept of the future. It describes multiple platforms that are in use at this time. Most of the engineered scaffolds are

created from nanoparticle assemblies, generally by chemical modification or by adsorption and subsequent collation of the nanoparticle-based fibers into sheets. Thus, the type of material used for nanoparticle construction affects the desired degree of "stiffness" or flexibility of the constructed tissue, which in turn dictates the usage of the engineered tissue product. For example, the "stiffness" of an engineered construct to be employed as a vascular graft in the repair of damaged arteries is different from that of the heart valves. This implies that multiple physical and chemical material engineering factors also affect the properties of the constructed engineered graft, besides the cellular and tissue matrix. Some of these factors can be controlled by regulating the process used for the construction of the nanofiber scaffold, while the selection of the nanofiber material itself also influences these characteristics. Before discussing specific polymers that are used for the construction of nanofibers which compose tissue-engineered scaffolds, disadvantages of such systems and possible strategies to overcome such issues are discussed next.

Integration of the bioengineered tissue with the rest of the organ is a significant step in achieving structural and functional harmony of the implant with the organ in question. For example, for the successful functioning of a cardiac prosthetic device, its revascularization and surface colonization by tissue cells would be a precondition for functional alignment. To elaborate further, the success of the nanoengineered scaffold depends on the ease with which it integrates into the electrical circuitry of the heart, without creating a separate rhythmic entity which would lead to the development of arrhythmia. Additionally, eventually the scaffold should develop contractility in rhythm with the heart. These features present specialized challenges for the construction of nanomaterial-based scaffolds, since multiple physical factors such as the ability to coat the surface of the scaffold, scaffold flexibility, and cell adhesion properties must be engineered into the platform. Some of these challenges can be addressed by the use of specialized polymers, or the use of specialized engineering processes during nanofiber development or, finally, the coating of polymers onto the device allowing surface modification that promotes some of the desired characteristics.

Generally, the choice of the manufacturing method affects the type of fibers that nanoparticles will produce. This in turn will have consequences for the scaffold. There are three techniques known as phase-separation, electrospinning and self-assembly which can be employed for the construction of nanoscaffolds. Some common polymers used for nanoparticle fabrication by the electrospray technique are listed in the Table 11.1 below.

Table 11.1 Common polymers used for the design of nanofiber scaffolds employing the electrospray technique.

Synthetic Polymers	**Natural Polymers**
Poly(l-lactic) acid	Collagen
Polyglycolic acid	DNA
ε-caprolactone	Silk

11.5 Injectable Biopolymers

11.5.1 Introduction and Historical Perspective

Over time, and with the advent of stem cell technology, the field of tissue reconstruction has gained prominence for cardiovascular repair and regeneration. These engineering processes are based on the appropriate and timely delivery of stem cells to the damaged heart tissue, such as may occur following a myocardial infarction where a part of the cardiac tissue undergoes necrotic death, accompanied by adequate volumes of polymers or other base materials that would enhance integration with the healthy myocardium to allow the reconstructive process to initiate. Even though this is a technically and technologically challenging approach, it is becoming popular for cardiac reconstruction due mainly to two reasons: 1) there is a paucity of endogenously occurring stem cells in the human heart, and 2) normal pathological processes would replace the necrotic cardiac tissue with fibrosis, further reducing the elasticity and contractility of the remodeled myocardium.

11.5.2 Cardiac Restructuring

Multiple techniques may be used to bolster and expedite cardiac restructuring that occurs following an adverse cardiac event. There are two primary approaches currently emerging: that of 1) *cellular cardiomyoplasty*, or 2) *cardiac tissue engineering*. Seeding damaged cardiac tissue with stem cells to allow for regrowth and repair following an episode of myocardial infarction or congestive heart failure is known as cellular cardiomyoplasty. However, injecting the stem cells in combination with biopolymers is an approach known as cardiac tissue engineering. A recent advancement in the field of surgical cardiac restructuring has been the parenteral delivery of both a biopolymer support matrix and stem cells either via direct injection into the myocardium or into the coronary vasculature. Interestingly, it was noted that an injection of a polymer matrix, such as alginate, without the inclusion of stem cells, resulted in cardiac remodeling following myocardial infarction [18]. Cardiac remodeling refers to the restructuring of the myocardium which usually occurs on a relatively larger and noticeable scale following adverse cardiac events such as myocardial infarction. During this well-known process cardiac tissue is replaced and/or realigned to maximize contractility, while persevering functionality in an otherwise compromised fibrotic heart muscle. Based on this premise, there has emerged a whole field of cardiac reconstructive surgery where injectable biological polymers are combined with stem cells and delivered to the damaged myocardium following episodes of cardiac duress. The ultimate aim of inducing such restructuring within the organ is to reduce the myocardial burden on the heart; more specifically, since the polymer matrix is assumed to behave similar to the normal, healthy myocardial tissue in terms of elasticity, its integration with the damaged myocardium is assumed to result in a larger myocardial mass and ameliorated end-diastolic volume [19], which in turn is assumed to result in lowered contractility as per the Frank-Starling law of the heart.

11.5.3 Select Biopolymer Agents Used as Bioinjectables in Cardiovascular Intervention

11.5.3.1 Alginate

Using a rat animal model of myocardial infarction, where the rat heart espoused new and old cardiac infarcts, Landa *et al.* reported the construction and injection of a new type of absorbable, Calcium-crosslinked, low-viscosity alginate biopolymer, which could be injected into the heart, but post-implantation would undergo phase transition into the hydrogel form. The use of this novel biopolymer was based on the premise that prevention of adverse restructuring in the heart may lead to improvements of cardiac functionality parameters or at least a preservation of current function, and restricted deterioration. Seven days after the induction of myocardial infarction in the rat, the authors injected this novel alginate biopolymer in a biotinylated form. They performed assessment studies using both post-mortem serial histological and serial echocardiography approaches in live rats. This study demonstrated for the first time that the decellularized version of the alginate polymer by itself was sufficient to prevent adverse cardiac remodeling by increasing scar thickness, since a majority of the alginate polymer was replaced by connective tissue over time in the infarct area following injectable delivery into the rat heart. The polymer seemed to prevent ventricular dilatation and the accompanying dysfunction by promoting mural cardiac thickening due to connective tissue-based recolonization of the affected region [19].

11.5.3.2 Collagen

Collagen is a connective tissue protein and one of the most abundant proteins in nature. As a protein, it is a natural biopolymer of amino acids and in the context of a bioinjectable for use in cardiac surgery post myocardial infarction or congestive heart failure, it is thought to prevent the movement of injected stem cells within the heart, keeping them restricted to specific regions. Similar to the work of the group discussed above for alginate, Danoviz *et al.* used a rat model of myocardial infarction to test whether collagen had any restrictive effects on stem cell migration within the negatively remodeled heart following an adverse event. The authors injected radiolabeled stem cells and collagen directly into the rat myocardium following myocardial infarction, harvested organs a day later and performed gamma-emission counting on them to determine the distribution of injected collagen and stem cells. They found that the collagen group with stem cells retained more cells in the myocardium at 26.8+/-2.4%, when compared with the control, stem cells combined with culture medium, but no polymer matrix, 4.8+/-0.7%. Separately, they conducted another experiment where they assessed the stem cell/collagen matrix 4 weeks after injection into myocardial infarction into the rat heart. The assessment was based upon direct morphometric measurement of the heart and showed that left ventricle perimeter and percentage of interstitial collagen was greatly reduced in collagen biopolymer group compared to media controls. Lastly, the group also conducted functional assessment under pharmacological stress and demonstrated that stroke volume (SV) and left ventricle end-diastolic pressure remained the same in the collagen group compared to the control [20].

Taken together, these studies suggest interesting and novel clinical uses of collagen as a bioinjectable polymer, particularly for achieving augmented cardiac function, while reducing the restructuring-induced losses of injected cardiac stem cells following myocardial infarction.

11.5.3.3 Matrigel

Matrigel is a compound polymer obtained from a mouse tumor cell extract. It is liquid at low temperatures, but gels at physiological temperature. In animal experiments, Kofidis *et al.* demonstrated the efficacy of using matrigel biopolymer to enhance cardiac function following adverse events. Using Balb-C mice following induction of myocardial infarction, they showed that in mice that received the matrigel matrix with green fluorescent protein (GFP)-labeled stem cells, the matrigel hardened at body temperature and upon histological examination two weeks after injection, showed "setting" in the exact shape of the infarct. The authors reported that the mice treated with the compound had a superior heart function compared with the controls ($P<0.0001$ by ANOVA/Bonferroni test; group I: 27.1+/−5.4, group II:11.9+/−2.4, group III:16.2+/−2.8, group IV: 19.1+/−2.7) [21].

11.6 Vascular Restructuring

Interestingly, such tissue-level restructuring is not restricted to the heart, but also extends to blood vessels such as arteries, where a number of vessel layers such as in the intima may be repaired and restructured following an adverse event. The notion of combined delivery of biopolymer matrix and stem cells to allow enhanced vascular restructuring and repair is an extension of the concept outlined above. In fact, the incidence of patients suffering from occluded arteriolar conditions that require surgical intervention is quite pronounced in the U.S. On average, yearly in the United States, somewhere around half-a-million arterial bypasses are routinely performed [22], which are interventions needed to resume blood flow in an organ where the arteries have been occluded as a result of disease or other causes.

11.7 Conclusions and Future Directions

In this chapter I have presented multiple examples of polymeric utilization in the field of cardiovascular intervention. Both synthetic and natural biopolymers are employed for the design and/or manufacture of devices that either directly brace anatomical structures or otherwise improve functionality or delivery of drug molecules or prevent negative cardiac restructuring following adverse events such as myocardial infarction or congestive heart failure. In fact a perusal of material presented here should form a cogent argument for the centrality of the use of polymeric materials for a variety of implants and devices. The durability, flexibility of functionality, ease and cost effectiveness of manufacture, and in many cases the relative biological inertness and consequent lack of toxicity make polymeric materials an excellent choice for cardiovascular devices. In particular,

the manufacture of artificial "organs" using polymeric nanoparticle platforms for the construction of tissue scaffolds is a promising area for research and development in the future. The incessant advent of modern science, coupled with the availability of novel, cutting-edge manufacturing technologies have created a demand niche for the discovery, characterization, and use of novel polymeric materials for use in pharmaceutical and medical formulations which will have a positive impact on global health, especially in the field of cardiovascular discovery.

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12

Polymeric Prosthetic Systems for Site-Specific Drug Administration: Physical and Chemical Properties

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Abstract

Polymeric materials having biomedical applications can be classified into two major groups according to their use: those employed in prosthetic devices, such as cardiovascular and orthopedic prostheses; and those employed as therapeutic systems, such as drug carriers. Among prosthetic systems, polymeric materials can be used as coatings or as cemented prostheses. Some of the major advantages in using polymeric materials for biomedical applications are their flexibility, biocompatibility, the possibility of tailoring their mechanical properties and their ability to incorporate therapeutic agents into their matrix in order to allow drug administration at a specific site. The aim of this chapter is to summarize the uses and applications in the field of medical devices, as well as to discuss the pharmaceutical, physical and chemical properties of two of the most popular biomedical polymers: poly(methyl methacrylate) and polyurethanes (PU). In particular, we will center our attention on their use as site-specific drug administration and their application in two great areas of prosthetic devices, bone tissue and cardiovascular engineering. We will also cover their use in diagnosis and in therapeutic treatments along with advances and future perspectives in both areas.

Keywords: Medical device, bone-tissue engineering, cardiovascular-tissue engineering, specific drug administration

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Abbreviations

Active pharmaceutical ingredients (API)
Antibiotic cement impregnated intramedullary nails (ACIINs)
Arginine-glycine-aspartic acid (RGD)
Bare-metal stents (BMS)
Camptothecin (CPT)
Contrast agent (CA)
Coronary artery bypass grafting (CABG)
Coronary artery disease (CAD)
7-tert-butyldimethylsilyl-10-hydroxy-camptothecin (DB-67)
Drug-eluting stent (DES)
Extracellular matrix (ECM)
Hidroxyapatite (HA)
In-stent restenosis (ISR)
Liquid-crystal displays (LCD)
Lysine diisocyanate (LDI)
Magnetic resonance imaging (MRI)
Methicillin-resistant Staphylococcus aureus (MRSA)
Methyl methacrylate (MMA)
Percutaneous osteoplasty (PO)
Percutaneous transluminal coronary angioplasty (PTCA)
Polyethylene oxide (PEO)
Polyethylene terephthalate (PET)
Poly(lactic-coglycolic acid) (PLGA)
Poly(methyl methacrylate) (PMMA)
Polytetrafluoroethylene (PTFE)
Polyurethane (PU)
Polyurethanes (PUs)
Self-assembled monolayers (SAMs)
Shape memory polymers (SMP)
Total hip arthroplasty (THA)
Total knee arthroplasty (TKA)

12.1 Introduction

Nowadays, active pharmaceutical ingredients (API) are only a part of the arsenal to face prophylaxis and treatment of diseases. The main goal of therapy is that the API gets to the right place at the right concentration, and it is then that pharmaceutical dosage forms and administration route are especially relevant. API can be incorporated into the body through several routes (oral, sublingual, intramuscular, subcutaneous) and each one has a specific purpose. Oral administration can be considered as one of the safest and least expensive routes, and these are the reasons why it is the most widely used. Nevertheless, it has several complications associated with the liberation

and absorption processes (gastric acid/enzyme degradation, first-pass metabolism, for instance).

On the other hand, although intravenous administration avoids problems associated with liberation and absorption, the pharmacokinetics still requires multiple doses to keep concentration almost constant in the therapeutic window. Moreover, all conventional pharmaceutical dosage forms have also the disadvantage that the drug is distributed systemically, which leads to adverse side effects [1]. Nowadays, the trend is towards the search of new systems to achieve a controlled local delivery by reducing systemic side effects and reaching therapeutic concentration.

This concept has impacted the development of new pharmaceutical dosage forms for drug delivery (liposomes, micelles, nanoparticles, etc.), as well as the modification of traditional medical devices, prosthesis and implants. The way to improve performance is to search for a specific controlled reaction with the tissues in order to induce a therapeutic response. Although the ideal material for human tissue repair should be the regenerated tissue itself, bioartificial fixing of human organs has been an area of great interest for medical researchers [2]. Replacing damaged tissue (bone, muscular and/or cardiovascular) by healthy human tissues may be more complicated than it seems, due to the inmunocompatibility between the host and the implanted tissue, which may lead to implant rejection and necrosis. As a consequence, biomaterials are a promising solution for circumventing these issues. It is important to note that the biomaterial to be used depends on the specific medical application and the desired purpose. These materials are the basic constituent of devices, prostheses or implants whose aim is to restore functions of organs and tissues, repair or heal nerves or tissue as well as to substitute tissue or osseous structures [3]. They have a wide range of applications, as shown in Table 12.1.

The first definition described a *biomaterial as a nonviable material used in a medical device, intended to interact with biological systems* [4]. Nowadays, this definition has evolved and takes in consideration many new topics, such as biodegradability and biocompatibility. In medical applications, biomaterials are rarely used as isolated materials, but are more commonly integrated into devices or implants.

The successful application of a medical device or implant in a clinical setting depends on the properties of the biomaterials used to fabricate the device or implant, along with numerous other factors, such as the manufacturing and processing history of the materials, and the specific application in which the device is used.

For instance, a biomaterial should obey some important features to be used in medical devices, prosthesis and implants, such as [5,6]:

- Biocompatibility;
- Sterilizability;
- Biostability over timescales;
- Biofunctionality during the implantation;
- Mechanical, electrical and physical compatibility;
- Morphological or topographical aspects (structural and surface compatibility with the host tissue);
- Adequate manufacturing.

Table 12.1 Biomaterials in medicine.

Application	Types of Biomaterials
Skeletal system	Titanium, Ti-Al-V alloys, Co-Cr alloys, stainless steel, poly (methyl methacrylate), hydroxylapatite, teflon, dacron, calcium phosphates
Cardiovascular system	Polyurethane, teflon, dacron, reprocessed tissue or treated natural tissues, stainless steel, carbon, silicone rubber, titanium,
Organs	Polyurethane, cellulose, silicone-collagen composites, polyacrylonitrile, silicone rubber, polysuphone, titanium
Ophthalmologic	Acrylates, poly (methyl methacrylate), polypropylene, silicone-acrylate, hidrogel, collagen
Others	
Breast implants	Silicone
Hernia mesch	Silicone, polypropylene, teflon
Blood bags	Poly(vinyl chloride)
Ear tubes	Silicone, teflon
Sutures	Polylactic acid, polydioxanone, polypropylene, stainless steel
Intrauterine device	Silicone, copper

Biomaterials could be classified by source, biological role, function (duration of use), composition or structure. According to their composition a biomaterial could be classified into four groups [7]:

- *Metals* (e.g., titanium and its alloys, Co-Cr alloys, stainless steels, etc.);
- *Ceramics* (e.g., alumina, zirconia, calcium phosphates, marine coral, etc.);
- *Polymers* (e.g., chitosan, cellulose, polilactic acid, polyglicolic acid, polyurethanes, poly(methyl methacrylate), etc.);
- *Composites* (e.g., Alumina (Al_2O_3), Zirconia (ZrO_2), hydroxiapatite, bioglass, etc.).

Each type of material has its own advantages that makes it suitable for each specific applications, but it is difficult for a single material to have all the required properties [8]. For instance, ceramics and metals are strong and tough materials suitable for joint replacements as well as dental, bone and orthopedic implants. However, Metals can corrode and are dense, while ceramics are brittle and non-resilient.

Composites, on the other hand, are used as joint implants and heart valves and have as an advantage that they are strong and tailor-made, but they are difficult to manufacture.

Finally, polymers are resilient and easy to fabricate. They are used in sutures, blood vessels, hip sockets, ear, nose and other soft tissues implants [7].

In this chapter, we intend to focus our attention on biocompatible polymers that allow drug administration at a specific site. A comprehensive review of its applications to medical devices, as well as a discussion of their physical and chemical properties is

provided. Specifically, we will discuss poly(methyl methacrylate) (PMMA) and polyurethanes (PUs) because of their low cost of manufacturing, their massive use, and simple chemistry. This chapter will be centered in their use as drug reservoir, and their application in two great areas: bone tissue and cardiovascular engineering. We will also cover future perspectives and challenges for applying them through other techniques besides the traditional ones, as well as for the incorporation of drug-eluting systems to other medical devices.

To address these specific topics, we will present a brief review of the general features of these different polymers used in medical devices.

12.2 Polymers Used in Medical Devices: General Features

A polymer is a very large, chain-like molecule made up of simple chemical units called monomers. It can be naturally occurring or synthetic. Polymers have a wide range of applications and can be classified into three major groups: biopolymers, polymers with hydrolyzable backbones, and polymers with carbon backbones [9,10].

Biopolymers are polymers formed in nature during the growth cycles of all organisms; hence, they are also referred to as natural polymers. Their synthesis generally involves enzyme-catalyzed, chain growth polymerization reactions of activated monomers, which are typically formed within cells by complex metabolic processes. Examples of biopolymers are:

- Polysaccharides
- Polypeptides of natural origin
- Bacterial polyesters

Synthetic **polymers with hydrolyzable backbones** have been found to be susceptible to biodegradation. In this group you can find polymers such as:

- Polyesters
- Polycaprolactone
- Polyamides
- Polyurethanes and polyureas
- Polyanhydrides
- Poly(amide-enamine)

Another category involves the **polymers with carbon backbones** which are not susceptible to hydrolysis, like vinyl polymers. Their biodegradation, if it occurs at all, requires an oxidation process. Most of the biodegradable vinyl polymers contain an easily oxidizable functional group. Approaches to improve the biodegradability of vinyl polymers often include the addition of catalysts to promote their oxidation or photooxidation [9,10]. This category includes:

- Poly(vinyl alcohol) and poly(vinyl acetate)
- Polyacrylates and polymethacrylates

12.3 Risks Associated with Surgical Procedures

Open and invasive procedures always have a risk of contamination, but the presence of biomaterials in surgeries increases the risk of infection due to their susceptibility to bacterial colonization [11,12].

Adhesion and colonization of microorganism on the prosthesis (either bone tissue or cardiovascular), mostly occurs during the intervention, which is the main reason why the major infections are of nosocomial origin (hospital acquiring infection).

When adhesion of bacteria occurs on the surface of the prosthesis an invasive inflammatory process can be developed. If bacteria proliferation continues, a biofilm is formed, and consequently a three-dimensional structure protects bacteria against the patient's own defense system and from systemic antibiotic therapy [13]. Bacteria do not grow exponentially, but rather exist in a slow growing or starvation state. These microorganisms can produce an extracellular matrix that protects them from a hostile environment, enabling them to evade the host immune system and antibiotic treatment. This biofilm facilitates bacterial survival under nutrient-poor, stressful conditions, which occur in the host, and results in structurally-complex, heterogeneous bacterial aggregates associated with a surface [14,15].

In the last decade, researchers have focus their efforts in the development of novel techniques which allow local antibacterial therapy. These techniques involve the use of antibiotic impregnated interim prostheses to treat infections. In order to maintain constant drug concentrations in the specific area of treatment, the device is impregnated with various antibiotics. The use of this method is a widespread technique in the treatment of postoperative infections. The advantages and benefits of these methods rely on the immediate treatment of the source of infection [16].

Organisms responsible for the majority of the infections present in a surgery are Gram-positive. Among the most common bacteria, we can mention *Staphylococcus aureus (S. aureus), S. Epidermidis* and *S.coagulase-negative.* These bacteria have become the leading cause of infections related to indwelling medical devices such as vascular catheters, prosthetic joints and artificial heart valves [17,18].

As we mentioned before, antibiotics are administered systemically as part of routine clinical therapy with the disadvantage that high doses are needed to reach effective concentrations at the infected area. Therefore strategies that limit initial bacterial adhesion and subsequent biofilm formation are needed. Such approaches typically involve either passive or active chemical modification of the device surface. Passive strategies include modifying the interfacial properties of an implant, such as hydrophobicity, by grafting polymer chains or forming self-assemble monolayers (SAMs) on the surface. In contrast to passive coatings, active strategies use molecules such as antibiotics or nitric oxide donors bonded to the surface or localized within a polymer coating [14,15].

The desired characteristics for an antibiotic are to have a wide antibacterial spectrum, to be an effective bactericide at low concentrations, high elution from the matrix for prolonged periods, thermal stability, low allergy risks as well as low influence on the properties of the biomaterial and low serum protein binding [16,19]. Additionally, it is commonly used in the powder form. The antibiotics that implement most of these conditions are the aminoglycosides and glycopeptides.

12.4 Applications in Bone Tissue Engineering

As it was previously mentioned, human body tissues and structures may suffer a variety of destructive processes, including fracture, infection and even cancer, causing pain and loss of function. Under these circumstances, it may be possible to remove the diseased tissue and replace it with some suitable synthetic material [20]. One of the most important applications of biomaterials in medicine are the orthopedic implant devices and the lost bone tissue replacement. In this sense, the most used polymeric biomaterial is PMMA. Due to its biocompatible nature and tuneable mechanical properties, it has been widely used as bone cements and as screws in bone fixation. This is one of the main reasons why PMMA and its derivatives have been successfully used in vertebroplasty and are the most common adhesive to anchor prostheses.

PMMA is a transparent thermoplastic synthetic polymer of methyl methacrylate. PMMA may be synthesized via emulsion polymerization, solution polymerization, and bulk polymerization (Figure12.1). When a radical polymerization is used, the obtained PMMA is atactic and completely amorphous.

PMMA is a versatile material because of its transparency and durability, and it has been widely used in a broad range of fields, such as lenses for glasses, panels for building windows, skylights, signs and displays, liquid-crystal displays (LCD), and furniture. Different methacrylate polymers are extensively used in medical and dental devices where purity and stability are critical to performance.

In a recent review, Arora *et al.* [21] described the historical background of PMMA bone cements, stating that in 1936 Heraeus Kulzer GmbH was at the forefront of bone cement technology after discovering that the dough formed by mixing ground PMMA powder and liquid monomer hardens when benzoyl peroxide is added and the mixture reaches a temperature of 100°C. This PMMA mixture was first used clinically in 1938 in the repair of cranial defects in monkeys. These same researchers [21] also informed that modern acrylic bone cement technology started in 1943 when Degussa and Heraeus Kulzer GmbH described the polymerization of MMA at room temperature with a co-initiator, such as a tertiary aromatic amine, which was added to the previous mixture. The first ones to use this technology were the dental surgeons for dental fixatives and fixtures [21,22]. Afterwards this same acrylic cement was used for a total hip arthroplasty, in order to anchor the prostheses. Since these experimental procedures were carried out for the first time, the use of PMMA cement bone has become widespread among orthopaedic procedures. In particular, antibiotic loaded bone cements have been used in joint replacement to provide short- to medium-term protection against prosthetic infection since 1970. The idea was to overlap and eventually replace the

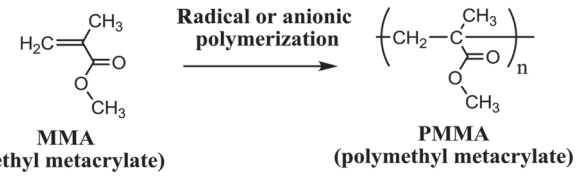


Figure12.1 Synthesis of PMMA.

prophylaxis provided by peri-operative intravenous antibiotics, which are released in high concentration, enough to exceed the minimum inhibitory concentration of potential colonizing bacteria [23].

The most important application of PMMA in biomedical technology is in the field of bone cements; however it is also used as coating for metallic devices and intramedullary nails. This is based on the idea that some of the desirable characteristics for a permanent implant are osteointegration, very high biocompatibility and corrosion resistance. Most implants are made of metals due to their strength and resistance. The main metallic biomaterials are stainless steels, titanium, cobalt alloy, and titanium alloys. However, metallic materials lack bioactivity; therefore, the incorporation of coatings into orthopedic implants seems to be an effective strategy in order to modulate the surrounding environment of the implant. The coating must satisfy several important properties, such as biocompatibility (no adverse tissue response), the promotion of osteoblasts growth and proliferation, and also, it is desirable for a coating to be able to induce cell differentiation into osteogenic cells. Researchers have focused on different features to design and develop useful coatings to achieve the properties mentioned above. Some of these features are surface roughness, porosity, and the incorporation of elements to encourage growth tissue.

12.4.1 Surgical Applications of PMMA

Antibiotic-loaded bone cements and coated prostheses are used in many orthopedic surgeries, diminishing the high risk of infection due to the invasiveness and aggressiveness of the procedures. There are different types of prostheses depending on the specific surgery: they can be temporary (spacer) or permanent; have different shape and function; and they can be made of just PMMA or a material combination. In the following section we will describe the use of antibiotic-loaded PMMA bone cement in four different surgical procedures: vertebroplasty and kyphoplasty, osteoplasty, total joint arthroplasty and bone fracture repair. In addition, the properties and mechanism of action of most of the used antibiotics agents will be described.

12.4.1.1 Vertebroplasty and Kyphoplasty

Vertebroplasty and kyphoplasty are minimally invasive techniques whose aim is to stabilize vertebral compression fractures and provide immediate pain relief with minimal risk. These procedures consist in the percutaneous injection of the PMMA cement into the vertebral body, thus avoiding the morbidity and mortality associated with open surgery [24]. Radiograph images of percutaneous vertebroplasty are shown in Figure 12.2 [25].

Generally, vertebral compression fractures are a consequence of osteoporosis, a disease characterized by a decrease in bone mass and a micro-architectural weakening of bone tissue. Considering that vertebral column is a complex structure whose aim is to provide mobility, flexibility and support to the upper part of the human body along with protection to the spinal cord; maintaining as well as restoring it to its natural shape is essential. In particular, the vertebral body bears up to 20% of the compressive force supported by the human body [26]. Computed tomography scans of fractured

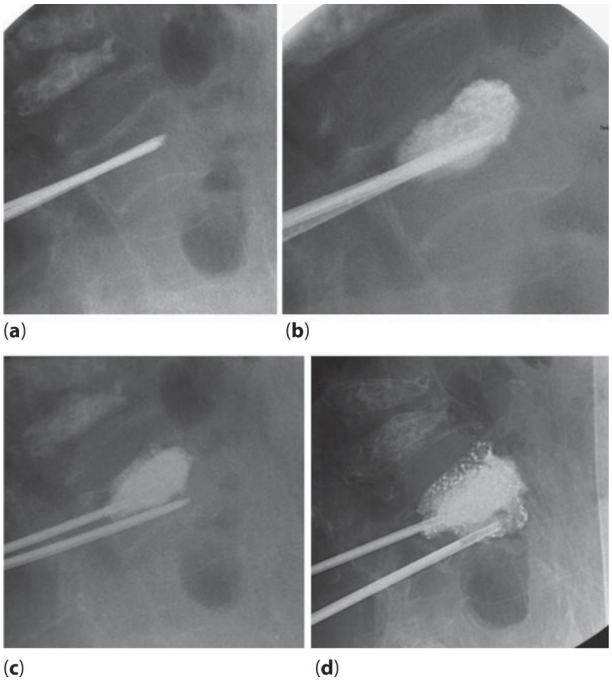


Figure 12.2 Percutaneous Vertebroplasty: Needle insertion into the mid-part of the vertebral body (A), and cement injection (B, C, D). Reprinted with permission from [25]. Copyright 2012 Elsevier.

vertebrae before and after reconstruction with percutaneous vertebroplasty are shown in Figure 12.3 [27].

Taking into account that both procedures are minimally invasive, bacterial infection is rare. However, some cases have been reported [28–30], and several authors have suggested the use of antibiotic-loaded bone cements in order to prevent this complication [31].

12.4.1.2 Osteoplasty

Osteoplasty, on the other hand, is a branch of orthopedic surgery related to bone repair or bone grafting, where acrylic bone cement is also used to reduce pain and improve mobility. This procedure is generally related to lesions caused by metastatic bone disease or Giant cell tumor, which is a rapidly growing tumor that generally occurs at or near the ends of long bones in young adults [32]. Bone is one of the most frequent sites for the spread of many common cancers such as breast, prostate, lung, and kidney, and bone is also usually affected in multiple myeloma. When appropriate systemic treatment for the underlying cancer fails, patients are considered for specific treatment. The injection of bone cement into a painful bone lesion refractory to conventional therapy (radiotherapy, chemotherapy, and narcotic analgesia), could determine immediate bone structure consolidation, reduce the risk of a pathological fracture, achieve pain regression, and improve mobility [33]. The mechanism of pain relief in patients with neoplastic lesions treated by percutaneous osteoplasty is not well known. Stabilization

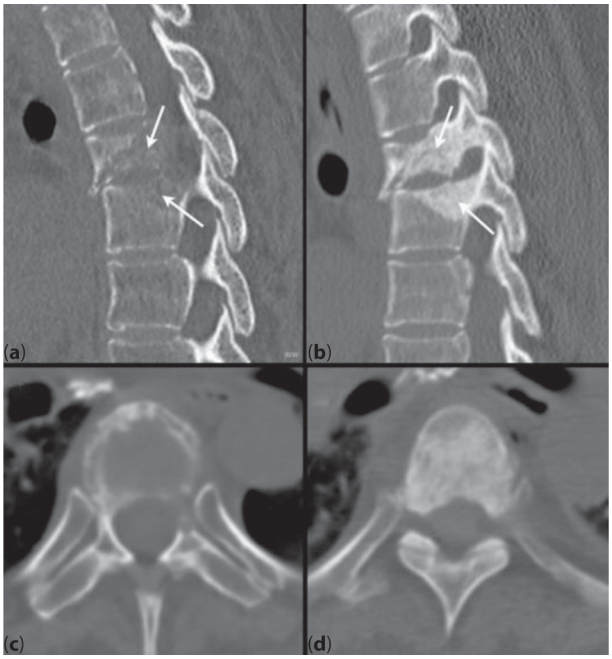


Figure 12.3 (A) Sagittal and (C) axial computed tomography (CT) scans of the thoracic spine performed 6 weeks before the attempted vertebroplasty, demonstrating the presence of pathological compression fractures because of lytic lesions at T5 and superior end plate of T6 (white arrows). (B) Sagittal and (D) axial CT scans of the thoracic spine performed after the attempted vertebroplasty, demonstrating complete remodeling of the vertebral bodies (white arrows). Reprinted with permission from [27]. Copyright 2014 Elsevier.

of the affected bone in addition to the analgesic effect of PMMA due to the thermal effect itself seems to be the main contributors for short-term pain relief. Long-term pain relief presumably was offered by reducing the tumor volume and long-term control of the tumor.

Giant cell tumor is locally aggressive, commonly destroying upwards of 50% of the bone area, thus leading to a treatment which generally involves surgery of the affected limb; where 20–30% of the cases are treated by amputation. A proposed alternative to amputation is a complete curettage, i.e., surgical scraping. However the treated area is left unstable and unsupported, and tumor recurrence as high as 78% has been reported. In this sense, curettage and cementation with PMMA bone cement has been proposed in order to achieve structural stability and decrease giant cell tumor recurrence. This apparent drop in tumor appearance is attributed to high polymerization temperatures causing tumor cells necrosis [34,35].

Taking in consideration that osteoplasty is generally related to the appearance of tumors in bone tissue, the use of chemotherapeutic drug-loaded bone cement is being evaluated. In this sense the same principles for antibiotic-loaded bone cement are applied. Several studies have been carried out in order to determine the elution profile of different drugs with potential or proven antitumoral activity. Zwolak *et al.* [36] studied both the release profile and cytotoxic effect of zoledronic acid-loaded bone

cement, as this drug is known to reduce osteoclast activity. More recently, Handal *et al.* [37] investigated both the elution profile and the mechanical properties of methotrexate-loaded bone cement, a well-known chemotherapeutic drug. Their most recent research is focused on the improvement of the elution profile of this same drug when soluble fillers are added to the formulation, as the main problem of these drug-loaded bone cements is that 80%–90% of the drug remains in the polymer matrix [38]. Other chemotherapeutic agents such as doxorubicin and cisplatin, along with the previously mentioned methotrexate, have been studied in order to determine their efficiency when added to acrylic bone cements [39–41]. Nowadays, these types of drug-loaded bone cements are not commercially available.

12.4.1.3 Total Joint Arthroplasty

This procedure is used to reconstruct and restore the function of a damaged joint by the use of a prosthetic device. It consists in the removal of the injured cartilage and bone from the joint and their replacement with prosthetic components, which simulate the shape and movement of the joint. This kind of surgery has a high risk of infection due to exposure and the insertion of a foreign body, leading to a periprosthetic infection. Generally, periprosthetic infection is eradicated with one or more staged revisions. The method of choice depends on the patient and the circumstances [42].

In a one-stage procedure of an infected arthroplasty, the infected implant is removed, the compromising tissue is debrided, and a new prosthesis is inserted during the same operative session; with a systemic antibiotic treatment after surgery [43].

A two-stage procedure consists of the removal of infected implant, tissue and the cement during the first stage, followed by the insertion of a temporary spacer. In the second stage, after infection has healed, the spacer is removed and replaced with the definitive prosthesis.

Total joint arthroplasty has become the operation of choice in most cases of hip and knee problems [44], it remains the most effective and common treatment for eradication of infection, showing an infection control rate between 80% and 95% [16,45,46].

Historically, the use of antibiotic-loaded bone cement to prevent and treat orthopedic infections occurred in 1970 by the hands of Buchholz and Engelbrecht [47]. In 1976, Merck started the commercial production of gentamicin-PMMA beads with the name of Septopal, which were medically used in Germany. During the following years several studies were performed in order to evaluate the efficiency of PMMA antibiotic-beads in the prevention and treatment of bone infection [48].

Antibiotic-impregnated PMMA beads are surgically placed in the infected bone cavity to both sterilize and maintain dead space [49,50]. The advantages of using PMMA beads lies in a decreased systemic toxicity and adverse effects, due to the fact that there is no systemic antibiotic serum concentration. It also decrease the time the patient must stay in the hospital. Although high local concentration of antibiotics is desired since it minimizes systemic toxicity, there may be unfavorable effects on bone healing and regeneration. Some *in-vitro* studies showed that high concentrations of gentamicin, tobramycin, cefazolin, and vancomycin on osteoblasts decrease replication and eventually led to cell death when high concentrations were used [51,52]. Among other issues, the use of PMMA as a delivery vehicle for antibiotic beads has several limitations. Atibiotic-loaded

PMMA beads are non-biodegradable and therefore must be removed [50,53]. As mentioned before, this requires a second surgery to remove large quantities of beads. Also, the pharmacokinetic profile of antibiotic release from PMMA beads is not ideal.

In addition, the use of antibiotic-loaded spacers has emerged as the standard of care for prevention and treatment of postoperative infections. This method seeks to improve the failures arising from the use of Antibiotic-loaded PMMA cement beads. Some of the advantages corresponding to the use of spacers are: local treatment of infection due to the application of high levels of antibiotic, limitation of scar formation, preservation of joint mobility and tissue length, and the possibility of an easy re-implantation.

Many kinds of spacers have been developed with different form and function. The spacers can be divided into articulated or static (block spacer) [54,55]. Concerning the material, in most cases they can be made entirely of cement, a combination of cement components with other components made of plastic and metal, or be a re-sterilization of a prosthesis partially coated with antibiotic-loaded PMMA [54,56]. Besides, they can be industrially made, hand-made with bone cement sometimes performed in the operating room and also by the use of special molds [57–61].

Infection after total hip arthroplasty (THA) is one of the most severe complications in orthopedics. In the work of Regis *et al.* [58], an aggressive, antibiotic-resistant septic hip arthritis was eradicated by the implantation of an industrial preformed temporary spacer (Figure 12.4). Molded spacers also showed good results in the treatment of hip

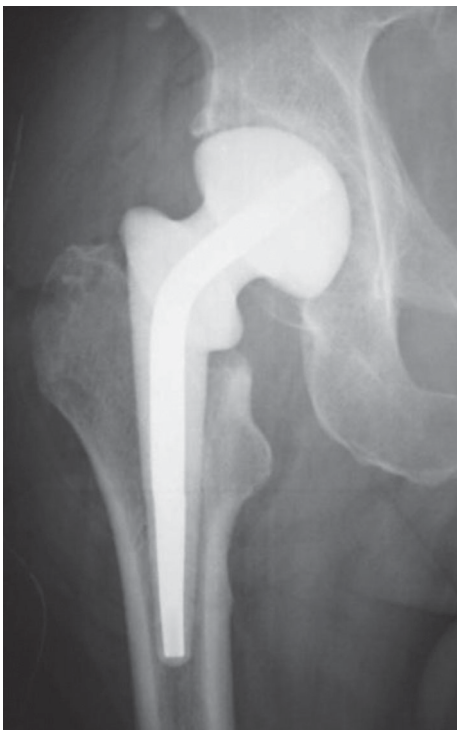


Figure12.4 Postoperative X-ray assessment showing the antibiotic-loaded spacer inserted in the femoral canal. Reprinted with permission from [58]. Copyright 2010 Elsevier.

infection. Kent *et al.* [62] describe a smooth, articulating, molded cement spacer with a central metal endoskeleton that can be inexpensively fabricated intra-operatively without the requirement of special equipment. A rush pin of adequate diameter for size of mold was inserted in the commercial cement spacer mold, and held in place using three drawing-up needles passed transversely through the mold. The mold was removed after the cement was introduced and polymerized. Results show a successfully eradicated infection in all treated cases.

In the case of total knee arthroplasty (TKA), block spacers have showed inferior results concerning postoperative range of motion and pain, compared with articulating spacers. This complicates second-stage surgery due to shortening of the quadriceps and ligaments, scar formation and tissue adherence. Researchers have demonstrated a better re-implantation and suppression of bone loss, relating to the use of articulating spacers. However, there is no significant difference in re-infection or complication rates between articulating and static spacers [63]. Shen *et al.* [64] described cement articulated spacers that were fabricated by mold and used for TKA infection treatment. A custom mold was made intra-operatively with bone cement and the standard posterior stabilized TKA provisional components, which were of the same size as the original prosthesis (Figure 12.5). Therefore fabrication of the spacers did not increase the total surgical time. Results show very low rates of re-infection and complication.

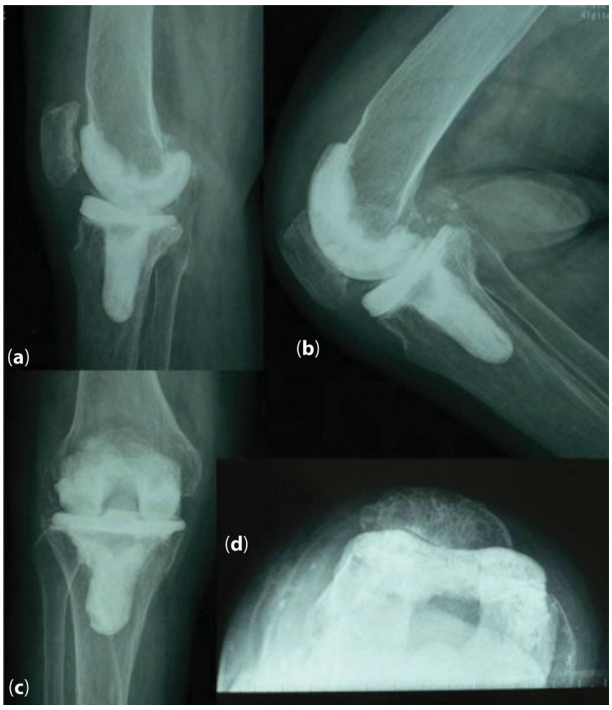


Figure 12.5 Lateral radiographs of active full extension (A) and flexion (B) illustrate the articulating spacer *in situ* with good range of motion; Anterior-posterior projection of the articulating spacer (C) *in situ* shows good alignment and medial-lateral balance; The spacer is also incongruent with the retained patella (D). Reprinted with permission from [64]. Copyright 2010 Elsevier.

Even though articulating spacers reduce hospital stays, industrially made spacers require a system of molds and instrumentation which is sometimes traduced in high cost incurrence [65]. However, common to all kinds of antibiotic-loaded spacers is the benefit to deliver high levels of antibiotic together with systemic treatment. In conclusion, due to the diversity in patient cases, the choice of spacer depends on several factors; such as, soft tissue state, degree of bone loss, antibiotics type, and financial and technical limitations.

12.4.1.4 Bone Fracture Repair

Antibiotic-cement coatings can be used in different kinds of bone fractures, such as tibia, femur, etc. Usually when a fracture of long bones occurs, the treatment consists of the insertion of a rod (usually called intramedullary nail) in the intramedullary cavity. In case of infection, the treatment commonly includes removal of the nail, debridement and lavage of compromising tissue, and the use of a fixator at non-union site, and sometimes the use of antibiotic-loaded cement beads or rods [66].

With the same goal of prevention and treatment of infections; several researches have evaluated the efficacy of producing coating nails. Gentamicin and vancomicyn have gained popularity in combination with PMMA due to their stability at body temperature, heat stability during cement polymerization, and water solubility to allow the diffusion of antibiotic from PMMA, among others properties. There have been many *in-vitro* studies on the diffusion or elution of antibiotics from PMMA bone

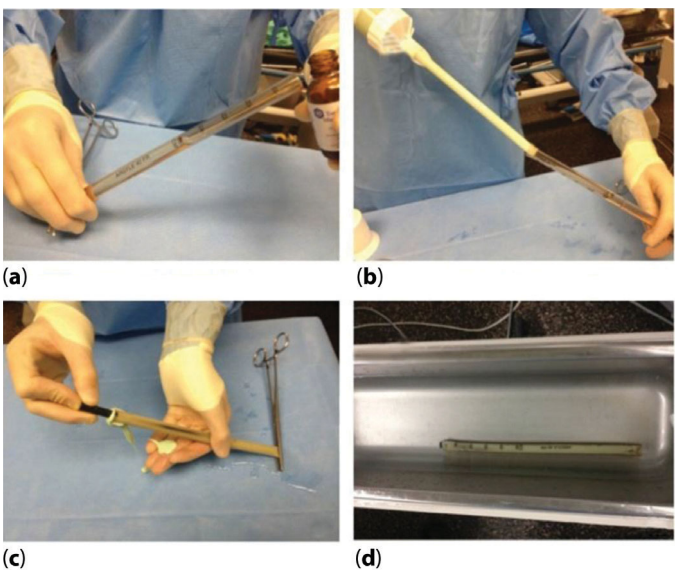


Figure 12.6 Preparation of the antibiotic nail. Loading the antibiotic nail: With one end of the chest tube closed with a Kocher (A), sterile mineral oil is poured to coat the inner surface of the plastic tube. Using a cement gun to fill the chest tube mold (B), the mineral oil is allowed to leak out under pressure. The nail is inserted into the antibiotic-impregnated cement (C), once placement of the nail is adequate, the mold is placed in a cool sterile saline bath (D) to prevent the plastic from melting during the exothermic stage. Reprinted with permission from [70]. Copyright 2014 Patient Safety in Surgery, BioMed Central.

cement [67]. Giavaresi *et al.* [68] evaluated a gentamicin-vancomycin-impregnated PMMA coating nail as a drug delivery device to treat bone and intramedullary infections. The researchers used methicillin-resistant *Staphylococcus aureus* (MRSA) to induce femoral osteomyelitis in a number of rabbits. The animals were divided into four groups and submitted to different treatments. Histology showed that the group in which a gentamicin-vancomycin-impregnated PMMA nail was inserted a marked improvement of the bone injuries compared with the other groups was observed. These results can lead to MRSA infection healing after surgical debridement and immediate implantation.

The use of antibiotic coating nails seems to be an efficient method for treating infection fractures. Several investigators have implemented a simple way to prepare a cement coated nail using a chest tube (Figure 12.6). This technique allows the treatment of the infection via local delivery of antibiotics without the need for expensive custom molds [69,70]. Dhanasekhar *et al.* [71] reported the treatment of 18 cases of infected nonunion of femur and tibia by means of antibiotic cement impregnated intramedullary nails (ACIINs), coated with the use of a chest tube. A mix of gentamicin, vancomycin and bone cement was used to fill the chest tube, followed by the insertion of the nail when the cement reaches doughy consistency. Among the cases, 11 were open fracture and 7 were closed. Finally infection was controlled in all cases.

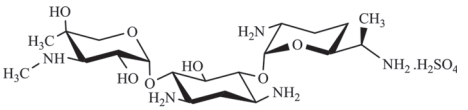
12.4.2 Antibiotic Treatment Commonly Used in Orthopedic Procedure Involving PMMA Bone Cement

Gentamicin sulphate (Figure 12.7) is considered as the antibiotic of choice for the local treatment of the infections mentioned above, given its wide antimicrobial spectrum activity, its excellent water solubility, its thermal stability and its low allergenicity, along with the fact that, gentamicin could still be found in tissues surrounding the implant for over 5 years, conferring long-term protection [15].

Gentamicin sulfate belongs to the aminoglicosidic antibiotic family, and is effective against many strains of Gram-negative and some strains of Gram-positive bacteria. Gentamicin does not have a uniquestructure, as it consists of five types which differ on its functional groups. Chemically, they are polycationic amino sugars produced by *Streptomyces sp, Micromonosporasp and Bacillus sp.*

They essentially show a dual mechanism of action: firstly, the antibiotic penetrates cells by active transport, altering the permeability of the membrane (which explains the synergy with β-lactams). Secondly, once it is present into the cell cytoplasm, it binds to the 30 S ribosomal subunit inhibiting protein synthesis.

Due to the increasing resistance of most staphylococci to gentamicin, other alternative antibiotics such as vancomycin, tobramycin, clindamycin, fusidic acid, cefamandol,



Gentamicin sulfate

Figure12.7 Chemical structure of gentamicin sulfate.

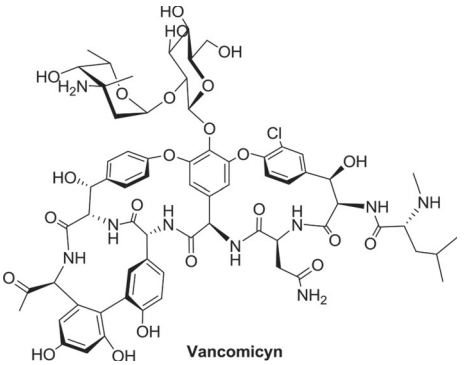


Figure 12.8 Chemical structure of vancomicyn.

cephalothin, and carbenicillin have been considered. Whenever gentamicin cannot be used, the choice is vancomycin (Figure 12.8). It belongs to the glycopeptides antibiotic class and is effective mostly against Gram-positive bacteria. Vancomycin is primarily used for the treatment of serious infections and when resistance to other antibiotics is suspected. Vancomycin is frequently administrated because of its antimicrobial activity against MRSA.

Gentamicin and vancomicyn are the antibiotics most widely typically used for the treatment of bone infections. Sometimes a combination of antibiotics used, for instance, rifampicin together with clindamycin or with gentamicin, showed a broader range of action when compared with monotherapy. As an alternative, these combinations can be advantageous, avoiding the likelihood of resistance development [72,73].

12.4.3 General Drawbacks of Antibiotic-Loaded Bone Cements

12.4.3.1 Thermal Behavior

Nowadays, commercially available acrylic bone cements are sold in a kit of two components, a solid phase (polymer powder with initiator) and a liquid ampoule containing the methyl methacrylate (MMA) and a tertiaryamine. This is based on the fact that polymerization of PMMA can also be initiated by oxidation-reduction reactions. An advantage of a redox initiator is that the radical production starts at lower temperatures in comparison to the traditional initiators, where a high temperature is necessary to generate the radical initiator. Generally, the most used combination includes benzoyl peroxide and *N*,*N*-dimethylaniline. The proposed initiation mechanism is shown in Figure12.9.

When mixing the two components, the polymerization of the liquid monomer starts to yield a solid mass. As the redox reaction is exothermic, a high level of heat is generated, leading to a temperature increase [74]. Due to the thermal behavior of the curing reaction of the bone cement, it is not possible to load the cement with every drug. It is mandatory for the drug to be thermally stable.

Sometimes this exposure of bone to high temperatures leads to incidences of bone necrosis and tissue damage that could, finally, result in failure of the prosthetic fixation [74]. It has been shown that three factors can affect bony tissue as a consequence of implant fixation using PMMA bone cement [75,76];

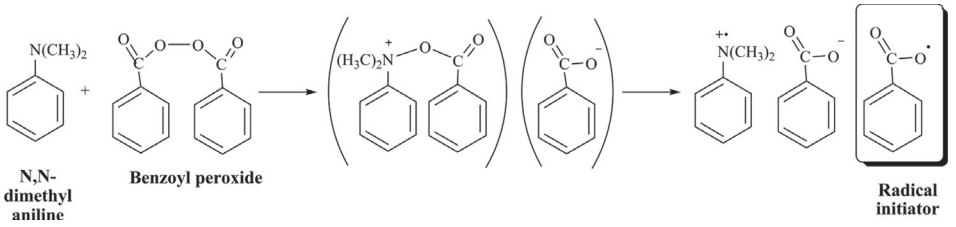


Figure 12.9 Radical formation in a redox-initiated polymerization.

- Polymerization temperature of bone cement;
- Cytotoxicity of the liquid monomer;
- Diminished vascularity of the bone due to surgical reaming of the medullary canal.

It has been cited by DiPisa *et al.* [77] that the temperature at the bone-cement interface is a function of:

- The quantity of heat produced by the bone cement;
- The rate at which the heat is produced;
- The thermal conductivity and thermal capacity of the bone, prosthesis and the cement;
- The initial conditions of the bone/cement prosthesis system, including initial and ambient temperature and preparation of the cement.

12.4.3.2 Mechanical Performance

One of the major concerns when considering the alteration of the basic formulation of PMMA bone cements is the effect over its mechanical performance. Although mechanical failure by aseptic loosening may be caused by thermal necrosis due to the amount of heat released during polymerization, and as a consequence of the bioinert nature of PMMA, which does not resorb or allow bone replacement, the mechanical behavior of PMMA bone cement plays a fundamental role in the success of the surgical procedure. In fact, most researches and new developments are mainly focused on the improvement of PMMA bone cement mechanical behavior in order to match the one of cortical and cancellous bone. Much has been written about modifications of the polymer matrix and the use of nanotechnology to overcome these drawbacks of PMMA bone cements [78]. Basically, the addition of a radiopaque agent which allows real-time monitoring of the medical intervention renders the material fragile due to poor compatibility between them and the polymer matrix. Usually, the dispersion of inorganic fillers is heterogeneous, thus creating clumps where initiation of cracks may occur. However, inorganic particles act as a heat reservoir, diminishing the amount of heat released during polymerization reaction and hence lowering the maximum temperature reached and monomer evaporation. Monomer evaporation and liquid- and solid-phase mixing processes are the main causes of bone cement porosity. From the physical point of view, this is one of the most important factors controlling the mechanical performance of bone cements [79], as hollow inclusions formed in the cured cement increases the possibility

of cement fracture and causes a resultant loosening of the implanted prosthetic device or destabilization of the repaired bone structure.

Many authors have reported that the most common way of failure in PMMA bone cements is brittle fracture. This means that very little or no plastic deformation is found beyond the elastic region where a linear relationship exists between the stress and strain [80]. For brittle materials, flexural strength is an important mechanical parameter, as it represents the highest stress experienced within the material in its moment of rupture. A high degree of porosity in bone cements results in a decrease in the flexural strength of the material. From several studies it was concluded that voids act as stress raisers and render the material susceptible to early failure, i.e., cement with a high level of porosity also possessed poor compressive strength [81]. Generally, the overall mechanical behavior improved when a less porous material is obtained (less monomer evaporation occurred when vacuum mixing was used) [82].

Opposite to what is proposed and in order to improve the mechanical performance of acrylic bone cements, drug elution from this polymeric matrix needs a high level of porosity and interconnectivity. Release of antibiotics or any other drug from PMMA bone cements is highly dependent upon porosity based on the need for penetration of dissolution fluids into the polymer matrix, which additionally depends on the wettability of the bone cement surface [83,84]. It can be said that antibiotic release strictly depends on cement porosity and wettability, but it is not clear whether this is just a surface phenomenon or if it also involves the bulk [11,85]. An improved elution can be achieved by mixing antibiotics. As an example, tobramycin and vancomycin act synergistically when eluted from bone cement [86,87]. Even though using a liquid antibiotic greatly improves the elution profile compared to its powder form, the ultimate compressive strength is highly decreased due to a possible plasticizer effect of the liquid drug. This could be the reason why the powder form of antibiotics is more commonly used in antibiotic-loaded bone cements [88,89].

The use of antibiotic-loaded bone cements requires an exhaustive study of the balance between the importance of the mechanical performance and the need for high doses of drugs to treat infections.

12.4.4 PMMA Modified Materials

12.4.4.1 PMMA Modified with Biomolecules

A strategy for the design of new biomaterials consists of modifying PMMA. This could be made by hydrolyzing some methyl ester groups in a basic medium. The implant surface could be biologically functionalized with arginine-glycine-aspartic acid (RGD) peptides, a class of cellular adhesion factors, by reaction of the amine with the acid of PMMA using carbodiimide chemistry [90]. The PMMA modified surface now has the ability to bind cultured osteoblasts in high levels and show high proliferation rates *in vitro*. Schaffner *et al.* [90] reported that results of *in-vivo* studies of animals showed that the newly formed bone tissue generated when in direct contact with the RGD-peptide coated implants, while uncoated implants were separated from newly formed bone tissue by a fibrous tissue layer, thereby preventing the formation of a direct implant-bone bonding. The focus of this work was to induce tissue integration in bone implants.

A different approach involves binding the biomolecule to an acrylate anchor and grafting it to the PMMA backbone using UV-irradiation. Ito *et al.* [91] immobilized

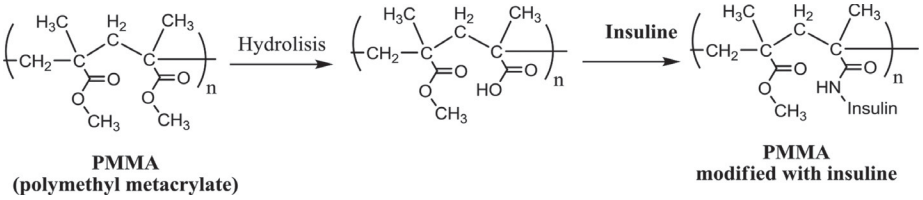


Figure12.10 PMMA modified with insuline.

insulin in PMMA films (Figure 12.10); this insulin activated the insulin receptor and downstream signaling proteins for long periods. The authors showed that the immobilized-insulin film could be used repeatedly without marked loss of activity.

Ito *et al.* [91] demonstrated a simple way of immobilization of biomolecules, such as insulin and opened the way to the biomaterial application. They proposed that biomaterial design with the use of biological signalling molecules could yield important advances in cell culture systems. The related application is in the production of artificial tissues and organs.

12.4.4.2 PMMA Modified Bone Cement for Hyperthermia Treatment

Hyperthermia is a technique used for cancer treatment and consists in the exposure of the body tissue to high temperatures. Researchers have shown that high temperatures can damage and kill cancer cells, usually with minimal injury to normal tissues. By killing cancer cells and damaging proteins and structures within cells, hyperthermia may shrink tumors. There is much research in which authors use a magnetic material which can generate heat when exposed to alternating magnetic field, based on the fact that magnetic materials under a variable magnetic field dissipate magnetic energy as heat, due to the continuous relaxation of the magnetization vector [92].

For instance, Matsumine *et al.* [93] mixed magnetite (Fe_3O_4 nanoparticles) with calcium phosphate-based cements for the treatment of tumors in femur, fibula, humerus or tibia, giving good clinical results. Kawashita *et al.* [94] reported the use of PMMA-based bone cements containing magnetite for hyperthermia treatment of cancer. The advantage of using PMMA cement instead of calcium phosphate cements lies in the better mechanical strength and uses of PMMA in percutaneous vertebroplasty. They can efficiently disperse these nanoparticles (up to a 60 wt%) in the cement matrix using PMMA powder and MMA monomer. They use benzyl peroxide as an initiator and *N*,*N*-dimethyl-*p*-toluidine as an accelerator. The increased concentration of magnetic nanoparticles increased the setting time. This might be a consequence of the polymerization reaction of MMA in the cements. This reaction would be inhibited by the addition of a large amount of nanoparticles, giving the increased observed in the setting time. On the other hand, they observed a decrease in temperature during the setting reaction. This might be attributed to the longer setting times.

12.4.4.3 PMMA Modified Bone Cement Used in Diagnosis

Magnetic resonance imaging (MRI) is useful in the planning of spinal cancer surgery because tumors, metastases, and especially pathologic fractures of the spine are clearly

visible on MRI. This technique makes feasible the quantification of the tumor infiltration in the surrounding tissues. It also allows determining the age of the fracture. The detection of soft tissues and other delicate structures, such as nerves, vessels, and spinal cord, improves the safety of these interventions [95]. Fast interventional MRI-sequences generate short repetition rates and allow real-time imaging during the intervention.

Wichlas *et al.* [96] developed a PMMA-based signal-inducing bone cement with hidroxyapatite (HA) and gadolinium-based contrast agent (CA) for the use in MRI interventional procedure. The CA enhances the MRI signal of the PMMA-HA compound. The use of HA-based bone substitutes relies on the fact that HAs contain water for MRI signaling and have an osteoconductive potential [97]. They also provide a biological bone cement interphase [98]. The mixture with PMMA cements increases the bioavailability of PMMA [99]. Wichlas *et al.* [96] demonstrated that the cement produces a clearly detectable MRI signal which equals the compressive strength of the existing cements for spinal cementoplasty. The signal intensity of the MRI cement can be manipulated by changing the amount of HA, the amount of CA or pulse sequence.

Bone is one of the most frequent sites for the spread of many common cancers such as breast, prostate, lung, and kidney, and bone is usually affected in multiple myeloma. Bone metastases and their skeletal related events are not controlled in ~20 to 40% of cancer patients, and the current therapeutic regimens leave up to 45% of patients with inadequate or undermanaged pain control. Percutaneous osteoplasty (PO), the injection of bone cement into a painful bone lesion refractory to conventional therapy (radiotherapy, chemotherapy, and narcotic analgesia), could determine immediate bone structure consolidation, reduce the risk of a pathological fracture, achieve pain regression, and improve mobility [33].

12.5 Applications in Cardiovascular Tissue Engineering

Cardiovascular tissue engineering aims to create functional tissue scaffolds that can re-establish the structure and function of injured sites of the cardiovascular system. A considerable amount of cardiovascular therapeutics, particularly for major and serious disorders, involves the use of devices. Some of these may be implanted by surgery, whereas others are inserted via minimally invasive procedures involving catheterization. Although different synthetic materials have been used for the development of cardiovascular devices, PUs have several advantages over the others. Besides their biocompatibility and their mechanical flexibility [100–103], PUs have very high flexural endurance compared to most elastomers, making them prime candidates for cardiovascular implants.

Polyurethane (PU) is a polymer composed of a chain of organic units joined by carbamate (urethane, -NH-COO-) bonds. It was first described by Otto Bayer and his coworkers in 1947 [104]. PUs can be applied in many areas because of their characteristic properties (biocompatibility and mechanical flexibility) and because of the great variety of building blocks that can be incorporated. They are widely used in industry as durable elastomeric wheels and tires; automotive suspension bushings; high performance adhesives; surface coatings and surface sealants; construction materials [105]; synthetic fibers and in pharmaceutical and biomedical devices [106]. The properties of the PUs can be tailored by selecting the proper building blocks in order to get a material with special characteristics such as bio-inertity and biodegradability. For instance, bioinert PUs are used in medical

devices and artificial organs because they have excellent chemical stability, resistance to abrasion and mechanical properties [107]. In contrast, biodegradable PUs are used as implants for tissue repair and as drug delivery systems [108].

The traditional synthesis of PUs involves a step polymerization between a di- or poly-isocyanate with a diols/polyols. A linear polymer is obtained if di-functional isocyanates and polyols are used (see Figure 12.11). Both the isocyanates and polyols used to make polyurethanes contain on average two or more functional groups per molecule.

The PUs are synthesized using three components: diisocyanates, polyols and chain extenders (diols or diamines), as shown in Figure 12.11. A chain extender is used to improve the mechanical properties of the polymer by connecting the hard (composed by -NH-COO- and chain extender) and soft (polyol part) segments of the PUs (Figure 12.12). Generally a low molecular weight diamine or diols are used to accomplish this function.

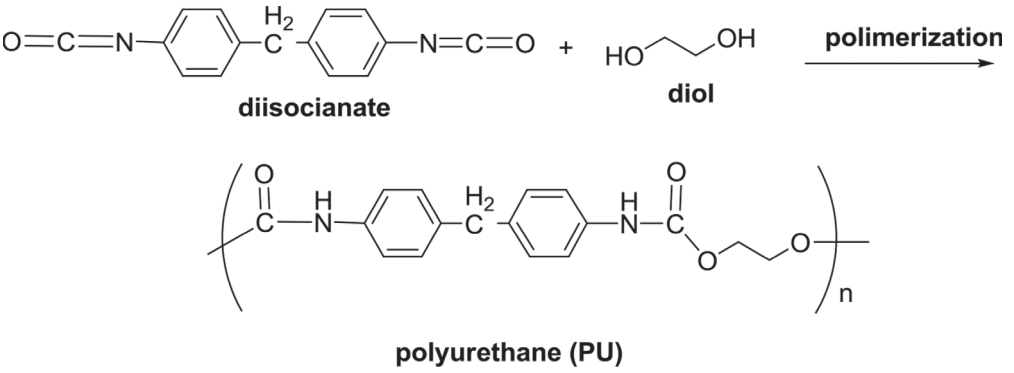


Figure 12.11 Synthesis of polyurethanes.

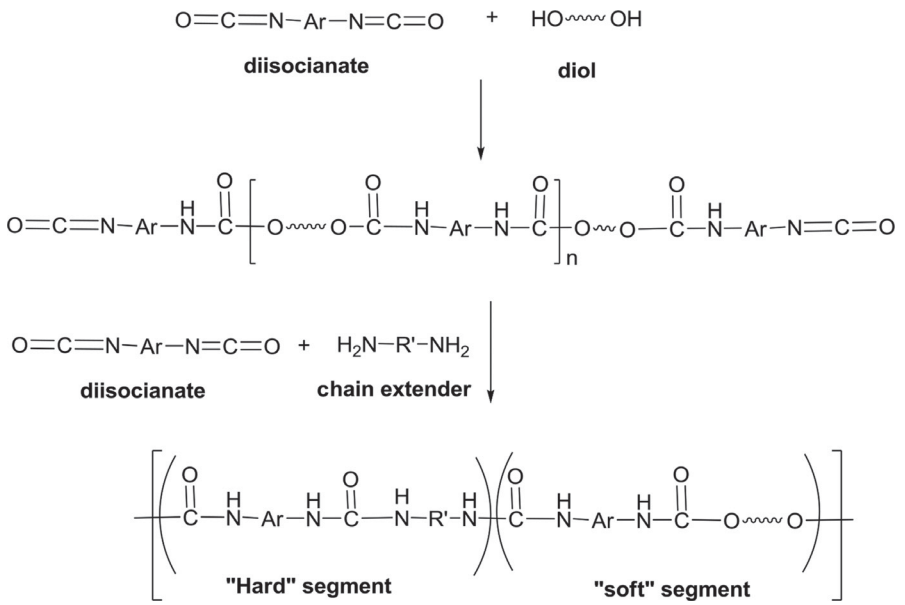
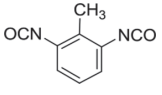
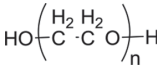
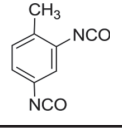
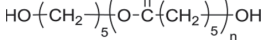
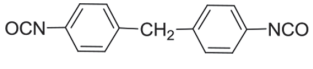
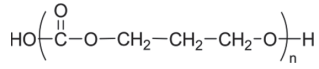
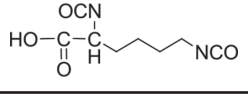
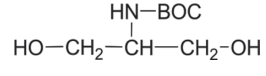
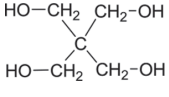
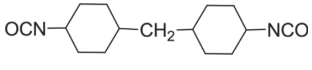


Figure 12.12 Polymerization of PU with a chain extender.

Table 12.2 Diisocyanates and polyols most commonly used for the synthesis of PUs.

Diisocianates		**Polyols**	
Name	*Structure*	*Name*	*Structure*
2,6- toluendiisocyanate		Polyethylene oxide	
2,4- toluendiisocyanate		Polycaprolactonediol (PCL)	
4,4'-methylenediphenyl diisocyanate (MDI)		Poly (trimethylene) carbonate	
Lysine-diisocyanate (LDI)		N-BOC-serinol	
1,6-hexamethylene-diisocyanate		Pentaerythritol	
4,4'-methylene bis(cyclohexylisocyanate)			

Aromatic diisocyanates are more reactive than aliphatic ones, the PUs derived from the first ones tend to undergo photodegradation, while the second ones are more resistant to the UV irradiation [109]. The properties depend greatly on the isocyanate and polyol used in the polymerization. For instance, an elastic polymer is obtained if a long and stretchy section of a polyol is used. A rigid and tough PU is obtained due to a large number of cross-linkages in the polymer. Long chains and low crosslinking gives a polymer that is very flexible and malleable, but in contrast, a hard polymer is obtained when short chain with lots of ramifications is used. In Table 12.2, the most common polyols and diisocyanates used for the PUs synthesis are listed.

Polyurethanes obtained from renewable resources, such as carbohydrates, have attracted much attention in recent years [110,111]. This new interest results from great biomass access and their potential as replacements for some petroleum-based polymer precursors. The polymers obtained from natural products are expected to be biodegradable and non-toxic. The inclusion of hydrophilic monomers, such as carbohydrates, into the polymer chain facilitates water attack, increasing the hydrolytic degradation.

The first cardiovascular developments based on polyurethanes date back to the 1950s, when a polyurethane film was inserted between solid Teflon® fixation rings with semilunar extensions for prosthetic valve replacement [112]. Since then different approaches, such as drug incorporation, have been attempted in order to overcome the difficulties associated with the developed materials, such as stenosis, emboli, calcification and thrombosis, and infections associated with surgical procedures. Nowadays, the research on cardiovascular implants based on polyurethanes technology includes the development of drug-eluting stents, vascular grafts and catheters among others.

12.5.1 Cardiovascular Devices

Many different devices have been used for cardiovascular therapy, from vascular catheters to electronic pacemakers and artificial implants. Some of these devices have been improved, adding the possibility to deliver drugs in the injured site.

Intra-arterial catheters have been used for different objectives, such as the placement of other devices like stents, the delivery of drugs to various targets in the cardiovascular system and the delivery of embolic materials to close arterial-venous fistulas. Drug therapy has also been combined with catheter ablation, pacemakers and cardioverter defibrillators in order to treat arrhythmias. On the other hand, implants for the reconstruction or functional replacement of cardiovascular components have been combined with drugs to prevent thrombosis. Finally, drugs to avoid restenosis have been widely employed in different devices such as drug-eluting stents.

Among the many existing drug-eluting devices used for the treatment of cardiovascular issues, we will focus on vascular stents, angioplasty balloons and vascular grafts, describing the importance of the localized drug delivery in each case.

12.5.1.1 Vascular Stents

Coronary stents are placed during a percutaneous coronary intervention procedure, also known as an angioplasty, usually as a treatment for coronary artery disease (CAD). CAD is commonly characterized by atherosclerotic obstruction of vessels responsible

for providing adequate blood supply to the myocardium and is one of the major causes of death and disability in the developed world [113,114].

Atherosclerotic plaque is a complex lesion which is a result of an inflammatory and reparative process. The atheroma plaque contains extracellular deposits of calcium salts, blood components, cholesterol crystals, and acid mucopolysaccharides. The initial changes, however, seem to occur at the cellular level, often accompanied by an abnormal intracellular storage of lipids, particularly cholesterol esters, fatty acids, and lipoprotein complexes. The rupture of the plaque can generate thrombosis, reductions in the vessel lumen and consequently in the cardiac perfusion, and acute or chronic consequences.

Percutaneous transluminal coronary angioplasty (PTCA) is used to widen the stenosis of the coronary without an open surgical procedure. During this procedure, a catheter with a small inflatable balloon on the end is positioned within the narrowed portion of the artery. As the balloon is inflated, the atherosclerotic plaque is compressed and the wall of the artery is stretched, dilating the stenosed segment of the artery. This procedure is usually followed by insertion of a stent at the site of stenosis. Figure 12.13 schematically shows the steps of this procedure.

The first developed stents were bare-metal stents (BMS), and were thought of as a solution to the risk of early abrupt closure, late restenosis due to elastic recoil, constrictive remodeling and intimal hyperplasia after the angioplasty procedure. Although the initial clinical results after BMS implantation are generally favorable, re-narrowing of the treated artery due to in-stent restenosis (ISR) is commonly observed. ISR is caused by excessive neointimal proliferation within the stented segment and its diagnostic threshold is ≥ 50% stenosis [115].

Several drugs could help to prevent ISR; however systemic pharmacotherapy has failed to demonstrate a reduction in randomized clinical trials [116]. Local administration of pharmacologic agents directly to the site of injured arterial tissue results in an interesting alternative. Among the strategies for localized drug delivery to the vessel wall, endoluminal delivery can be achieved by introducing the desired drug into the stent body. A drug-eluting stent (DES) is therefore a very promising approach to prevent post angioplasty ISR. Different DES have been developed through the last decades, including first-generation DES, such as sirolimus-eluting (Cypher®) and paclitaxel-eluting stents (Taxus®), second-generation DES, such as zotarolimus-eluting (Endeavor®)

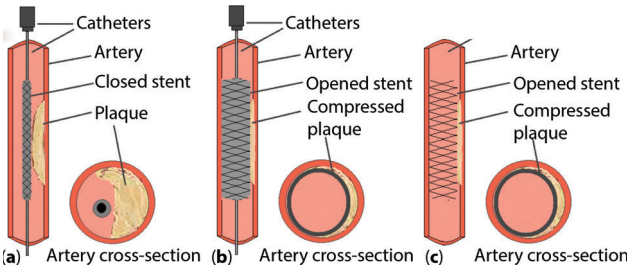


Figure 12.13 A) Deflated balloon catheter inserted into an artery narrowed by plaque. The inset image shows a cross-section of the artery with the inserted balloon catheter. B) The balloon is inflated, compressing the plaque against the artery wall. D) Widened artery with the stent holding the artery structure. The inset image shows a cross-section of the widened artery and compressed plaque.

and everolimus-eluting stents (Xience V®), and third generation DES, including those that use biodegradable polymers, polymer-free stents, and biodegradable stents.

Safety concerns have risen due to the observation of late stent thrombosis in the case of first-generation DES implantations. Second-generation DES have provided promising results compared with BMS and first-generation DES [117]. Newer third-generation stent technology has been evaluated in preclinical and initial clinical trials, but long-term data of large-scale randomized trials as well as registries comparing them to currently approved first- and second-generation DES are still lacking.

DES normally consists of three components: (a) stent platform, (b) stent coating and (c) therapeutic agent. The election of these components for the development of the ideal stent can be approached from three complementary perspectives: deliverability, efficacy, and safety. In order to achieve deliverability requirements, the stent should be a small device made of a flexible material, with a self-expanding nature. On the other hand, efficacy condition requires a uniform drug delivery rate, the required radial force to avoid recoil, and a lesion-specific stent configuration and disease-specific application. Finally, for the safety needs to be achieved, it should have a biomimicing biodegradable coating, with cell-specific drug action [118].

The stent platform is based on a mesh-like design to allow expansion, flexibility, and in some cases the ability to make/enlarge side openings for side vessels. Different metals have been used to develop these structures, such as 316L stainless steel, platinum-iridium alloy, tantalum, nitinol, cobalt-chromium alloy, titanium, pure iron and magnesium alloys [119]. Every material has its own pros and cons. A single material could not fulfill all the required expectations, so the other components of the stent body (coating and therapeutic agents) should be chosen in order to equate the weaknesses of the chosen metal mesh.

The coating materials for stents can be broadly classified into four types: inorganic materials, polymers, porous metals, and endothelial cells. In this work, we will focus on polymeric coatings, putting special focus on polyurethanes. The main function of the coating is to reduce the incidence of early and late stage thrombosis and restenosis during stent placement.

Different techniques have been used to coat stents, such as spray-drying, dip-coating and, more recently, electrospinning. The spray-drying method is a simple technique where the mixture of polymer and agent is dissolved in the organic solvent and then transferred into electrostatic spray machine. On the other hand, in the dip coating process, the metallic platform is dipped into a polymeric coating solution and then is withdrawn at a controlled speed. This second technique is often undesirable for coating complex geometries like stents. If coating solution gets entrapped in the device structure forming of a film across the open space between structural members of the device it could interfere with the mechanical performance of the stent and provide potential sites for platelet deposition [120]. On the other hand, electrospinning is a simple and innovative technique. In this process a solid fiber (composed of a viscous polymer solution) is produced by a generated electrical field. Subsequently, nanofibers are formed by continuous stretching, due to the electrostatic repulsion between the charged nanofibers and the evaporation of the solvent. Figure 12.14 shows a schematic illustration of the electrospinning setup needed for fabricating a stent covered with nanofibers. These electrospun fibers are optimal candidates for a wide range of important

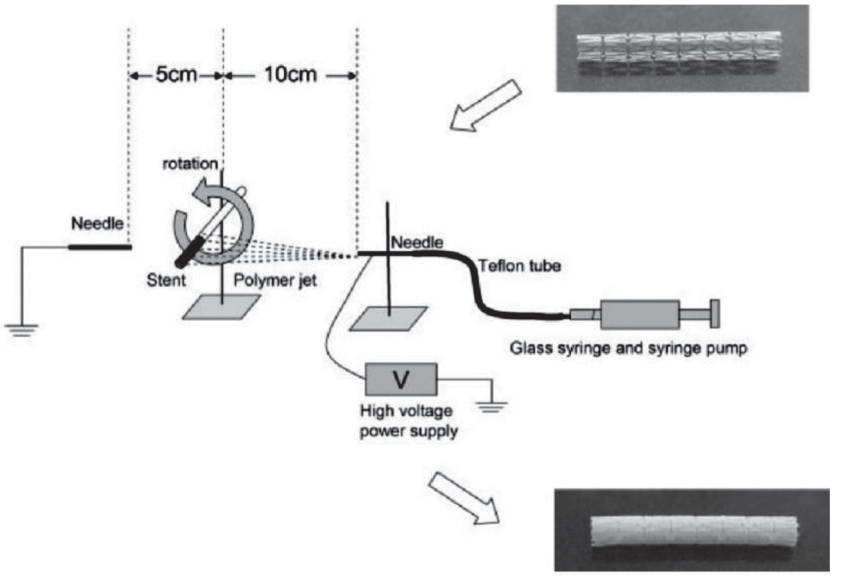


Figure 12.14 Schematic illustration showing the electrospinning procedure for fabricating a stent covered with nanofibers. A high voltage is applied between the needle and the ground to create an electrically charged jet of polymer solution. The polymer solution is jet dried to leave a polymerfiber on the stent. Reprinted with permission from [121]. Copyright 2009 Wiley.

applications in different areas such as drug carriers, tissue scaffolds, wound dressing, reinforcement materials, filters, protective clothing, electrodes, sensors, catalysts, etc. In this sense, nanofiber coatings obtained by the electrospinning technique have emerged as a novel structure for tissue regeneration, showing various advantages when compared with traditional coating approaches, as they hold great potential for mimicking the dimensions of extracellular matrix (ECM) [121]. Besides, compared to continuum coatings, electrospinning mats possess a high surface area which can be useful to improve drugs deposition and elution. Additionally, inside the stent, as turbulent blood flow can intervene with laminar flow, stents coated with nanofibers will provide steady and smooth flow-through, alleviating restenosis and plaque progression [122].

Among the most used synthetic polymers as cover materials for stent devices within the vasculature we can find polytetrafluoroethylene (PTFE), polyethylene terephthalate (PET), and polyurethane. PTFE is a synthetic polymer of tetrafluoroethylene $(C_2F_4)_n$ composed of a carbon chain saturated with fluorine. This polymer has hydrophobic properties conferred by the presence of electronegative fluorine atoms. This characteristic which results in unfavorable reactions that enhance platelet adhesion, activation and thrombus formation [123], consists of ethylene glycol $(C_{10}H_8O_4)_n$ and terephthalic acid. This polymer is susceptible to slow hydrolytic degradation resulting in unfavorable biological reactions, and is mostly used for larger diameter (≥ 10 mm) endovascular stent grafts. PET coatings are not suitable for small caliber vessels, given the strong inflammatory cellular response (development of a fibrous capsule, accumulation of giant cells and white blood cells and excessive extracellular matrix). This leads to a compacted fibrinotic luminal surface devoid of an endothelial cell lining that results in

narrowing of the luminal diameter, which poses a high risk of thrombosis and vessel occlusion [124].

As was mentioned before, PU has been extensively studied as a material for cardiovascular applications, and so far has provided encouraging data. The structure of PU consists of a hard segment that provides mechanical strength to the structure, combined with a soft segment that provides elasticity. *In-vitro* studies have shown that PU-covered stents have greater endothelial cell coverage when compared with PTFE, denoting its better biocompatibility [125].

Another advantage of PU compared to PTFE is the possibility to electrospin it. The electrospinning technique cannot be applied to fluorinated polymers such as PTFE due to its inherent insolubility in appropriate solvent systems. Kuraishi *et al.* [121] developed a novel covered stent employing electrospinning to deposit fine PU fibers onto stents, using the setup shown in Figure 12.14, finding encouraging results.

Finally, besides the metallic platform and the polymeric coating, drug incorporation and delivery are crucial characteristics of drug-eluting stents. The most usual mechanism used to incorporate the drug into the polymeric matrix is blending the drug with the polymeric solution to be used. In particular, published studies focusing on drug-eluting electrospun fibers for vascular applications use blend electrospinning approaches. These data demonstrate that (blend) electrospinning is a simple and versatile technique to incorporate various drugs into polymer fibers, while offering a localized and controlled drug release without significantly altering the morphology and mechanical properties of the ensuing fibrous constructs. A more detailed description of the drugs commonly introduced in the DES body in order to avoid ISR and thrombosis will be exposed in the next section.

12.5.1.2 Vascular Grafts

Another coronary procedure that is performed routinely around the world is coronary artery bypass grafting (CABG). Due to their excellent mechanical stability and their natural antithrombogenicity, patients own blood vessels are the most widely used conduits for coronary revascularization [126]. However there are a number of disadvantages associated with their use, such as the need for surgical harvest procedures, the heterogeneity in quality and size, and the limited availability, in some cases. Besides, late restenosis is usually caused by the inevitable damage of the vessel wall during the surgical procedure in CABG, which induces endothelial denudation, platelet adherence/activation, and leukocyte infiltration [127]. In order to overcome these problems, the scientific community is currently working on the development of an effective synthetic graft. Among the several advantages it would present, it can be found its unlimited availability, biomechanical uniformity and consistent quality and patency. Besides, a synthetic graft should be resistant to thrombosis and biocompatible, resembling a native artery, and have excellent biomechanical stability, being able to withstand the long-term hemodynamic stress of the arterial circulation. The possibility to incorporate drugs to the graft body could be an excellent approach to prevent late restenosis.

Synthetic materials used in the stents development such as PET or PTFE have failed in coronary revascularization [128]. Dacron grafts lead to thrombosis and neointimal thickening in low blood flow. The PTFE grafts also fail owing to surface thrombogenicity

for small vessels [129]. In this context, PUs have shown encouraging data as new raw materials for the development of synthetic vascular grafts with the possibility of delivering drugs locally.

New trends in vascular grafts development include the combination of PUs with other components. Ishii *et al.* worked on the development of a new vascular graft composed of a stable macrostructure and an absorbable microstructure. The authors developed a microporous polycarbonate-siloxane polyurethane graft that incorporates soluble collagen and hyaluronic acid into the pores [130]. In addition, a bi-layered scaffolding structure composed of poly(L-lactic acid)/bioresorbable segmented polyurethane blends for small-diameter vascular bypass grafts, was obtained by Montini Ballarin *et al.*[131] using the multilayer in electrospinning technique. They found the electrospun blends to exhibit better properties than raw polymers.

Different drugs have been incorporated into vascular grafts in order to treat the clinical complications associated with bypass grafting. The commonly delivered drugs together with their action mechanism will be described in the next section.

12.5.2 Drug Treatments Commonly Used in Cardiovascular Devices

Different drugs have been incorporated in cardiovascular devices in order to treat the clinical complications associated with the surgical procedures. After both stent implantation and bypass grafting, restenosis and thrombosis need to be prevented.

Currently, the traditional treatment for restenosis includes the treatment by anticoagulant/antiplatelet/antiproliferative agents, calcium channel antagonists, inhibitors of angiotensin converting enzyme, corticosteroids or a fish-oil diet [132]. First stages of treatment included the use of five drug antithrombotic regimens, such as aspirin, dipyridamole, dextran, and prolonged heparin followed by warfarin; heparin with aspirin being the association most widely used.

Nowadays, drug therapy aims to prevent surface mediated platelet activation through at least 12 months of dual antiplatelet therapy (aspirin and a P2Y12 receptor antagonist). Clopidogrel and tioclopidine are the most commonly used [133].

On the other hand, in order to avoid restenosis antitumoral agents with antiproliferative action have been included into the studied devices, like Paclitaxel (Taxol) and Sirolimus.

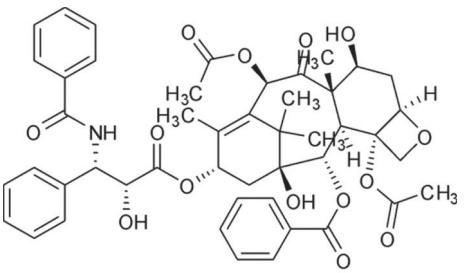


Figure 12.15 Chemical structure of paclitaxel.

Paclitaxel (Taxol®) is one of the best antineoplastic drugs found in nature in recent decades (Figure 12.15). It has excellent therapeutic effects against a wide spectrum of cancers. Moreover, recent studies have demonstrated the effectiveness of low-dose paclitaxel to treat non-cancer human diseases, such as skin disorders, renal and hepatic fibrosis, inflammation, axon regeneration, limb salvage, and coronary artery restenosis [134].

Paclitaxel has shown potent and sustained inhibitory effects on smooth muscle cell proliferation and migration in cell culture. This makes paclitaxel a very promising candidate for local drug therapy intended to address the proliferative and migratory processes involved in restenosis [135].

Paclitaxel, a natural diterpene alkaloid, was originally isolated from the bark of the Taxus brevifolia tree in the western region of the United States. It has high lipophilicity, low water solubility; high protein binding rate; and mainly disturbs the structure of the inner part of the cell membrane [134].

Paclitaxel belongs to the family of cytoskeletal drugs which target tubulin. Treatment leads to abnormality of the mitotic spindle assembly, chromosome segregation, and consequently defects of cell division. By stabilizing the microtubule polymer and preventing the disassembly of microtubules, paclitaxel arrests cell cycle in the G0/G1 and G2/M phases and induces apoptosis [134].

On the other hand, Rapamicyn (Sirolimus) is a natural product produced by a strain of *Streptomyces hygroscopicus*. Initially used as an antifungal agent with a high activity against candida, Sirolimus subsequently showed an important antitumor and immunosuppressive activity. Sirulimus has a unique mechanism of action, where molecular mechanism underlying the antifungal, antiproliferative, and immunosuppressive activities of sirolimus is the same. It forms an immunosuppressive complex with intracellular protein FKBP12 that blocks the activation of the cell-cycle-specific kinase, TOR, resulting in the blockage of cell-cycle progression.

Sirolimus has been impregnated into the microporous graft developed by Ishii *et al.*, creating a two-tiered drug-release system to promote patency. The drug-eluting graft showed excellent potency at 1 month and may encourage luminal endothelialization without excessive intimal hyperplasia. However, further long-term study would be needed to evaluate the efficacy of impregnated sirolimus [130]. Han *et al.* [136], on the other hand, used the electrospinning technique to develop vascular graft, including rapamycin (RM) in a polyurethane vascular graft, achieving an effective suppression of local smooth muscle cell proliferation. They found that RM release kinetics was characteristic of Fickian diffusion for at least 77 days *in vitro.* The RM-PU fibers generated via powder blending showed the highest encapsulation efficiency. The RM in grafts made of these fibers remained bioactive and was still able to inhibit smooth muscle cell proliferation after 77 days of continual *in-vitro* release.

Finally, in the case of grafts, antibiotics could be included. Unlike what happens in a stent implantation, where the surgery is performed through a catheter, bypass surgery is much more invasive, increasing the risk of infections. Some of the antibiotics with reported application for the treatment of graft infection are Sisomicin (SISO) and Vancomycin. SISO acts against *Staphylococci,* the major causative bacteria in graft infection, and it is also effective against *Pseudomonas aeruginosa*. Vancomycin, as was previously mentioned, is another potent antibiotic, which is being tried against methicillin-resistant *S. Aureus* [127].

12.5.3 Polyurethane Modified Materials

In the search for new drug-delivery systems, researchers found that they could introduce a biomolecule in the network of the PU. The use of a drug which contains reactive functional groups leads to a polymer in which the drug molecule is attached to the backbone of the polymer, and upon its degradation, the drug will be released in a controlled way, lowering the adverse effects of a locally high concentration of the drug and enhancing the ability to deliver the drug to the indicated place.

12.5.3.1 PU with Antibiotics and Anti-inflammatory Drugs

Hafeman *et al.* [137] synthesized a PU using hexametylene diisocyanate trimer and a polyester polyol, containing ε-caprolactone, glycolide and D,L-lactide, with tobramycin (Figure 12.16) as chain extender. This resulted in a PU network that upon degradation releases the drug over a long period of time.

Another approach was made by Abraham *et al.* [138], who attached p-aminosalicylic acid (Figure 12.17), an anti-inflammatory drug. The authors used a two-step protocol which involves first the grafting of hexamethylene diisocyanate onto a polyurethane matrix, and secondly the reaction of the isocyanate gropus with the amine groups of PAS.

12.5.3.2 PU with Immobilized Anticoagulant

As PUs are widely used in cardiovascular medical devices, it has been a goal to introduce anticoagulant molecules in the structure of the polymer by either chemical reaction or by grafting. For instance, means of achieving the introduction of heparin (Figure 12.18), a highly sulfated glycosaminoglycan widely used as an anticoagulant, in patients subjected to cardiovascular surgery, such as the introduction of stents or valves. The immobilization of heparin could be achieved in different ways, either by

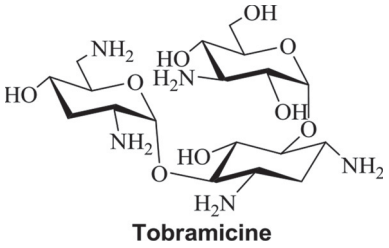


Figure 12.16 Chemical structure of tobramicine.

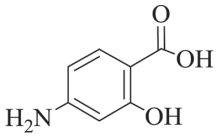


Figure 12.17 Chemical structure of p-aminosalicylic acid.

chemical reaction in which the heparin is used as a chain extender [139], or either by reaction of the side chains of PUs with the heparin molecule or by a graft polymerization [140]. One interesting approach was that of Ito *et al.* [141], who synthesized polyether urethane ureas containing tertiary amino groups in the side chains, which were modified by reaction with heparin. This polymer showed excellent antithrombogenicity. The authors showed that the release of heparin was slow, leading to a long-term antithrombogenic material. In this way, Kim *et al.* [142] immobilized heparin on the surface of polyurethane polymer by the use of coatings of a polyurethane-poly(ethylene oxide)-heparin graft copolymer, and a coating of thermosensitive polymers.

The promising results in blood compatibility of heparinized-PUs demonstrated that these new polymers reduce thrombus formation compared to nonheparinized PUs. Furthermore, the residual bioactivity of heparin was found to be approximately 25%, slightly higher than those reported elsewhere [143].

Other approaches with various antithrombotic drugs or molecules such as urokinase derivatives [144], prostacyclin [145], ADPase [146], dipyridamole [147], and hirudin [148] were developed, but none of them have *in-vivo* or clinical studies and are still being investigated.

12.5.3.3 PU with Antitumorals

Sivak *et al.* [149,150] developed biodegradable and biocompatible polyurethane foams for drug-delivery release of 7-tert-butyldimethylsilyl-10-hydroxy-camptothecin (DB-67) (Figure 12.19), based on lysine diisocyanate (LDI) and glycerol. Camptothecin (CPT) is a cytotoxic quinoline alkaloid, which exhibit anticancer activity in ovarian,

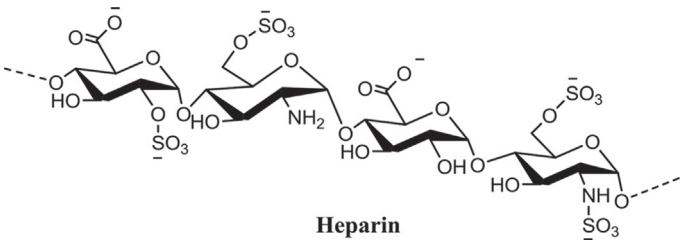


Figure 12.18 Chemical structure of heparin.

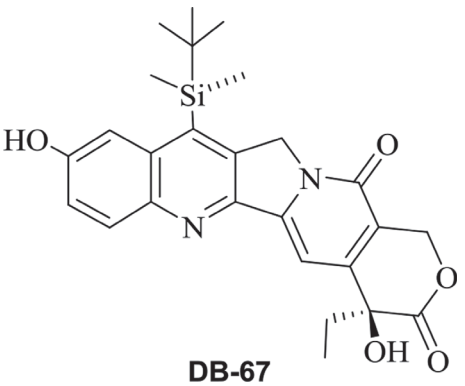
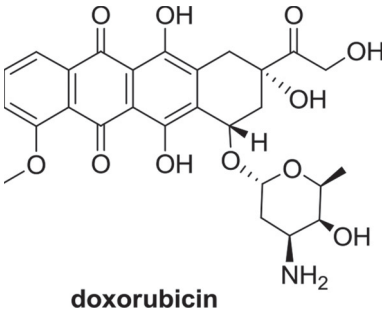


Figure 12.19 Chemical structure of DB-67.



doxorubicin

Figure 12.20 Chemical structure of doxorubicin.

breast, pancreas and gastric cancers [151,152]. The authors studied the impact of urethane catalysts (1,4-diazobicyclo[2.2.2]-octane, DABCO and 4,40-(oxydi-2,1-ethane-diyl)bismorpholine, DMDEE) on cellular proliferation in order to enhance the biocompatibility of the polyurethane materials. The foams synthesized in this way showed a potential use as drug-delivery devices because they were capable of delivering therapeutic concentrations of DB-67 *in vitro* over an 11 week test period. They also bonded DB-67 covalently into their delivery system by way of an organometallic urethane catalyst in order to get sufficient quantities to impact tumor growth and disease progression.

In this way, the Sivak group also synthesized a PU network using doxorubicin (Figure 12.20), another anticancer drug which contains several isocyanate reactive groups [153].

12.6 Future Perspectives

In the previous section some contemporary challenges in drug elution from polymeric matrices were explored, and recent works where PMMA and PUs have been used as a drug reservoir were detailed. However, there are a number of ways in which such composites could be prepared in order to improve the efficiency of the drug elution.

As was previously mentioned, the electrospinning technique is a novel methodology that allows the obtainment of micro- and nanofibers. The structures obtained from this technique have a huge potential as drug reservoir. Despite so far the electrospinning method has not been incorporated in orthopedic coatings, it could be properly used due to the important advantages electrospun mats have in front of a continuum coating.

We have previously discussed antibiotic-loaded cements produced by mixing the desired antibiotic with the PMMA cement. In this way, antibiotic elution and absorption are almost completely related to a surface phenomenon. Elution rates are dictated by several factors such as the porosity of the cement, surface area, wettability of the polymer, and the type and amount of antibiotic [1]. Increasing the porosity of the cement also increases drug elution [154]. A study by van de Belt *et al.* [83] showed that the initial release of gentamicin from an acrylic polymer matrix is partially a surface phenomenon, whereas the total amount released depends on bulk porosity. Therefore, a combination of surface roughness and porosity could improve the release kinetics of

drugs from bone cements. However, as we have seen in Section 12.4.3.2, a high increase in porosity of bone cements relies on poor mechanical properties. Most commercially available bone cements have a low efficiency in the delivery of drugs because the major proportion of the antibiotic is trapped inside the coating. Researchers have showed that less than 10% of the contained drug is eluted from a cemented coating [83,155–157]. For this reason, in order to improve antibiotic elution, new techniques need to be incorporated. Among these techniques electrospinning seems to be a good candidate because fiber mats can be produced with high porosity having interconnected pores and nanoscale matrix features [154,158–161]. Compared to continuum coatings, electrospun mats possess a high surface area, improving antibiotic deposition, elution, and also avoiding antibiotic agglomerations. Recently, Li *et al.* [162] have found a high elution rate of gentamicin from a coating of poly(lactic-coglycolicacid) (PLGA)/polyethylene oxide (PEO) produced by electrospinning technique. The researchers prepared a solution of PLGA/PEO with gentamicin to coat titanium implants. The release of gentamicin from the mat showed an initial gentamicin burst followed by a slow release, with a persistent antibacterial efficacy for 1 week and a significant reduction in the adhesion of the *Staphylococcus aureus.* In a similar work, Zhang *et al.* [163] formed a PLGA-vancomycin solution to cover a titanium implant surface by the electrospinning technique. *In-vitro* and *in-vivo* studies were performed. The release behavior of vancomycin from nanofiber coatings exhibited an initial burst on day 1, followed by a slow and controlled release over 28 days. In addition, *in-vivo* results exerted no cytotoxicity observed on osteoblasts, and also an active function of the vancomycin-coated titanium implants in treating implant-associated infection.

With the same idea of the previous works and taking into account the advantages of electrospinning technique, a possible solution to problems concerning antibiotics elution from PMMA cement coatings could be the development of electrospun mats with a controllable porosity. The mat and fibers morphologies obtained from this technique can be tailored in order to fulfill the needed characteristics by choosing different processes and solution parameters [161].

Besides mixing the antibiotic with the polymer solution, another deposition method should be taken into account. Deposition by layer can be an effective way to control antibiotic elution due to the possibility to choose the location of the antibiotic layer and the amount of drug by layer. In addition, the antibiotic agglomeration problems associated with continuum coatings could be resolved with the use of this deposition method.

In our research group, several studies are being performed in order to control antibiotic elution by both methods. Different devices are being tested to homogeneously deposit an antibiotic solution over an electrospun PMMA mat. So far, incipient results demonstrate that the use of electrospun mats with sprayed drugs as implant coatings may be a promising approach to improve elution efficiency.

These global findings suggest that electrospun fiber-based devices could be suitable to prevent and treat implant-associated infections.

On the other hand, the promising results obtained in drug delivery systems already employed in many pharmacological areas tend to encourage the expansion of these ideas into many other devices or prosthesis. The drug elution technology could be incorporated into different systems in order to improve the efficiency of the implanted device and/or prevent complications associated with the surgical procedure. An example of

devices in which drug elution property could be incorporated is heart valve devices. Heart valve disease is a cardiovascular affliction that may need surgical intervention as treatment. Currently, clinically available valve replacements are restricted to slightly modified mechanical and bioprosthetic valves. Polymeric heart valves could represent an attractive alternative to the existing prostheses, merging the superior durability of mechanical valves and the enhanced hemodynamic function of bioprosthetic valves.

Of all the materials tested, including collagen, silicone rubber, PTFE, and polyurethane, PUs have turned out to have the best biocompatibility, durability, and thrombogenic resistance. Polyurethane valve prostheses are either constructed from solvent-cast sheets of polyurethane, which are thermally formed into the correct leaflet geometry, or dip-cast valves, which use a stainless steel mold which is dipped into a polyurethane solution to produce the valve leaflets. It was found that the results for the dip-cast valve showed a more uniform distribution of mean axial velocity and Reynolds normal stress resulting from the more circular central orifice produced by the dip-cast leaflets [164].

Although polymeric heart valve applications are not yet optimal, mainly owing to their limited durability, researchers all around the world are working on the development of new prosthetic materials in order to overcome this difficulty. Taking into account the huge versatility of polyurethanes and the promising results obtained from drug-eluting stents and drug-loaded vascular grafts, future drug-eluting polyurethane heart valves will possibly be developed.

It is worth mentioning the role of nanotechnology in the development of improved medical devices and detection kits. In this sense, the introduction of magnetic particles in PMMA and PU matrixes in diagnostic techniques as well as treatments are widely studied. As an example, many researchers are focusing their attention on the design of shape memory polymers (SMP) for use in medical devices such as expandable stents and intravascular microactuators [165–167]. The key challenge of these new devices relies on designing a safe and effective method of thermally actuating *in vivo*. Buckley *et al.* [166] loaded a polymeric matrix with nickel zinc ferrite ferromagnetic particles to induce heat and demonstrated the feasibility of SMP actuation by inductive heating. It was shown that a rapid and uniform heating was achieved in complex device geometries and SMP recover the shape with no interference of the magnetic particles. The authors expect that with a further optimization of particle material that gives clinically acceptable Curie temperatures and magnetic field frequencies, enough heat will be gained to deploy SMP medical devices. The benefits reliant on inductive heating are, for instance, the elimination of power lines, the possibility of many complex devices shapes, the selective heating of specific areas; and as these new devices imply remote actuation by an external magnetic field, this allows tissue regeneration.

As mention before, the new perspectives are based on using the advantages of nanotechnology in the active coating for site-specific drug administration. In order to reduce bacterial adhesion and prevent biofilm formation, an increasing number of studies have focused their attention on developing antimicrobial agents with enhanced properties such as efficiency and controlled release. In this way, a great effort is set on designing coatings with nanoparticles combined with biocompatible polymers. Future goals involve the study of the effect of nanocoatings on healthy tissue as well as the

study of the drug release rate related to the nanostructure and the composition of the coating [8].

12.7 Conclusions

In the last few years, significant progress has been made in the field of polymeric prosthetic systems for site-specific drug administration. Due to inmunocompatibility problems, which are frequently found between the host and implanted human tissue, biomaterials emerge as a promising alternative to overcome these issues.

In particular, polymers are resilient and easy to fabricate and can be combined with specific drugs. Among many polymers, poly(methyl methacrylate) and polyurethanes are highlighted due to their biocompatibility and the possibility to be used in several medical devices. PMMA is usually employed in bone tissue engineering, while PU has applications mainly in cardiovascular tissue engineering. Both polymers can be designed for a specific application, and they have already been used in the development of prostheses with controlled drug release.

In this sense, new achievements have been made in the area of biomedical devices, where one of the most promising strategies involves the design of new biomaterials modified with biomolecules. These biomolecules can be attached to the structure of the polymer as described for the immobilization of insulin in the structure of PMMA. This approach is promising for the development of new artificial tissues and organs. Another interesting modification is the addition of magnetic particles to the PMMA matrix, as they are able to generate heat when exposed to a magnetic field. This new advance is promising as an application in hyperthermia treatments. In this sense, other modifications of the matrix in order to get new diagnostic and imaging techniques have been presented in the literature. For instance, some researchers used a PMMA matrix modified with hydroxyapatite and gadolimiun as a contrast agent, for use in magnetic resonance imaging. This is a very useful technique in order to detect and monitor bone consolidation, reduce the risk of a pathological fracture, achieve pain regression, and improve mobility.

Regarding PU, we can cite several new achievements related to this polymeric matrix. It is known that PU is more biocompatible and could be more biodegradable. PU has been modified with antibiotics and anti-inflammatory drugs. These drugs have been covalently introduced in the PU lattice, where these drugs were used as chain extensor during the polymerization reaction. It was shown that as PU degrades, it releases the biomolecule, leading to an antibiotic treatment over a longer period of time. A different approach was the immobilization of anticoagulants, such as heparin and antitumorals, as DB-67 or doxorubicine, in the net of the PU. These new advances are being studied in order to have new biomedical devices with new properties and applications.

Even though great advances have been made in the field of biomaterials for medical applications, as was mentioned before, the development of a polymeric matrix capable of acting as drug reservoir, which is also capable of having full control of the release rate of drugs, is still in its infancy. Biomaterials capable of replacing human tissue

and minimizing the risk of infections during surgery or avoiding problems, such as intrastent restenosis, will be obtained.

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13

Prospects of Guar Gum and Its Derivatives as Biomaterials

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Abstract

Guar gum is a natural nonionic polysaccharide that is nontoxic, biodegradable and biocompatible. It consists of a backbone of 1,4-linked β-*D*-mannopyranosyl units with branches of 1,6-linked α-*D*-galactopyranosyl units. Since guar gum has both primary and secondary hydroxyl groups on its backbone, it can be easily chemically modified to improve its physicochemical properties. In recent years, guar gum and its derivatives have shown promise as biomaterials for biomedical applications. This chapter is aimed at focusing on the recent developments and potential applications of guar gum and its derivatives as drug delivery systems, tissue engineering scaffolds, wound healing materials, biosensors and antimicrobial agents.

Keywords: Guar gum, drug delivery, biosensors, tissue engineering, wound healing

13.1 Introduction

Guar gum, an extract from the seeds of *Cyamopsis tetragonolobus,* shows a backbone of β-D-mannopyranoses linked 1→4 to which, on average, every alternate mannose and α-D-galactose is linked by 1→6 linkages, as shown in Figure 13.1 [1]. It contains about 80% galactomannan, 12% water, 5% protein, 2% acidic insoluble ash, 0.7% ash and 0.7% fat. As a natural biomaterial, guar gum is highly stable, safe, nontoxic, hydrophilic and biodegradable. Moreover, it has abundant resources in nature and has low processing costs [2]. Guar gum is soluble in cold water, hydrating quickly to produce viscous pseudoplastic solutions that, although shear-thinning, generally have greater low-shear viscosity than other hydrocolloids [3]. This gelling property retards release of the drug from the dosage form, and it is susceptible to degradation in the colonic environment [4]. Therefore, guar gum and its derivatives are widely considered as matrices for colon-specific drug delivery. Like other polymers used for colon targeting, guar gum can protect the drug in the stomach and small intestine environment, while delivering the drug to the colon where it undergoes assimilation by specific microorganisms or degradation by the enzymes, leading to the final delivery of the

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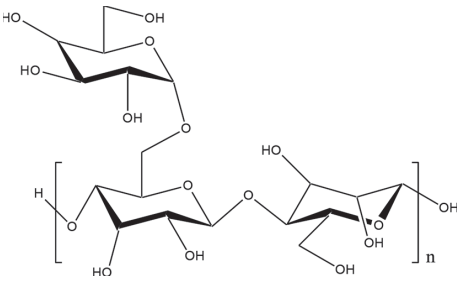

Figure 13.1 Structure of guar gum.

drug. Hence, it is used to form prodrugs, as a coating material or as a hydrogel entrapping drugs inside its network.

In recent years, hydrogels derived from guar gum and its derivatives have been considered as promising scaffolding materials for tissue engineering applications owing to their three-dimensional porous structure, biodegradability and biocompatibility, ability to imbibe a large amount of water, as well as biological fluids, and good mechanical integrity [5]. Such hydrogels can typically offer positive interactions with cells. Moreover, there has been a significant increase of interest in using guar gum-agarose polymer composite as an immobilization matrix for enzymes, living organisms, and cell carriers for the detection of various types of biomolecules because of an unusual combination of their properties, which includes an excellent membrane-forming ability, high permeability toward water, nontoxicity, low non-specific adsorption and good biocompatibility [6]. Guar gum and its derivatives have also been considered as tissue engineering scaffolds and antimicrobial agents due to their unique physicochemical properties. The aim of this chapter is to focus on the recent developments of guar gum and its derivatives as drug delivery systems, tissue engineering scaffolds, wound healing materials, biosensors and antimicrobial agents.

13.2 Developments of Guar Gum and Its Derivatives

13.2.1 Drug Delivery Systems (DDSs)

13.2.1.1 Colon-Specific DDSs

Guar gum and its derivatives could potentially be used as a matrix material for the preparation of colon-specific delivery systems of drugs. Guar gum-based matrix tablets of dexamethasone and other anti-inflammatory agents have shown very encouraging results as colon carriers. Matrix tablets of dexamethasone and budesonide were prepared using 60.5% w/w of guar gum in the tablet [7]. The study showed negligible drug release in simulated gastric and intestinal fluid, whereas in simulated colonic fluid significant increase in drug release was observed. The study demonstrated that the galactomannanase (> 0.1%) accelerated dissolution of dexamethasone and budesonide from guar gum matrix tablet. The extent of drug dissolution depended on concentration of galactomannanase. Rama Prasad *et al.* [8] prepared matrix tablet of indomethacin with

guar gum. These tablets were found to retain their integrity in 0.1 M HCl for 2 h and in Sorensen's phosphate buffer (pH 7.4) for 3 h, releasing only 21% of the drug in these 5 h. However, in presence of 2% rat cecal contents the drug release increased and further increased with 4% concentration of cecal contents. The drug release improved to about 91% in 4% cecal content medium after enzyme induction of rats. This study suggests the specificity of these matrices for enzyme trigger in the colon to release the drug. In the absence of enzyme system the guar gum swells to form a viscous layer that slows down the seeping of the dissolution fluid into the core. The initial 21% release can be attributed to the dissolution of indomethacin present on the surface of the tablet.

Guar gum has also been evaluated as a compression coating to protect the drug core of 5-amino salicylic acid in upper gastrointestinal tract (GIT) [9]. The tablets coated with 300, 200 and 150 mg of guar gum showed cumulative mean drug release percentages of 5.98 ± 0.70, 8.67 ± 0.35 and 12.09 ± 0.29 respectively after 26 h, while tablets coated with 125 mg guar gum disintegrated within 5 min in simulated gastric fluid. Cores with guar gum coat as high as 300 and 200 mg could not successfully release the drug in presence of rat cecal contents even in 26 h, as drug release was 23.85 ± 3.13 and 63.43 ± 6.30%, respectively. However, the formulation with 150 mg of guar gum as a coating showed 95.51 ± 1.50% of 5-amino salicylic acid release in presence of rat cecal contents after 26 h. Percent drug release from tablet increased considerably from 11th hour and the tablets were completely disintegrated in 26 h. The results of drug release studies on compression-coated tablets suggested that the thickness of guar gum coating in the range of 0.61–0.91 mm was sufficient to deliver the drugs selectively to the colon. Compression-coated tablets of 5-amino salicylic acid and matrix tablets of mebendazole have been prepared using guar gum as a carrier [10]. Matrix tablets containing various proportions of guar gum were prepared by wet granulation technique using starch paste as a binder. The tablets were evaluated for drug content uniformity, and were subjected to *in-vitro* drug release studies. The results of the studies showed that matrix tablets containing either 20% or 30% of guar gum are most likely to provide targeting of mebendazole for local action in the colon.

The pharmacokinetic evaluation of mebendazole containing guar gum matrix tablets against an immediate release tablet was carried out in human volunteers [11]. Six healthy volunteers participated in the study and a crossover design was followed. Mebendazole was administered at a dose of 50 mg both in immediate release tablet and colon-targeted tablets. On oral administration of colon-targeted tablets, mebendazole started appearing in the plasma at 5 h, and reached the peak concentration (C_{max} of 25.7 ± 2.6 ng/ml) at 9.4 ± 1.7 h (T_{max}), whereas the immediate release tablets produced peak plasma concentration (C_{max} of 37.2 ± 6.8 ng/ml) at 3.4 ± 0.9 h (T_{max}). Colon-targeted tablets showed delayed T_{max} and absorption time, and decreased C_{max} and absorption rate constant when compared to the immediate release tablets. The results of the study indicated that the guar gum-based colon-targeted tablets of mebendazole did not release the drug in stomach and small intestine, but delivered the drug to the colon, resulting in a slow absorption of the drug and making the drug available for local action in the colon.

The influence of concomitant administration of metronidazole/tinidazole on the usefulness of guar gum as a carrier for colon-specific drug delivery using guar gum matrix tablets of albendazole as a model formulation was investigated [12]. The results showed

that the release of albendazole from guar gum matrix tablets decreased with an increase in the dose of metronidazole and tinidazole administrated. From this study, it was observed that for successful design of colon-targeted delivery systems for drugs having antimicrobial activity against anaerobic bacteria using guar gum as a carrier, it requires a tight control of drug release until the swollen guar gum formulation is acted upon by colonic bacteria. In another study, matrix, multilayer and compression-coated tablets of metronidazole containing various proportions of guar gum were prepared for colon-targeted drug delivery [13]. Release studies showed that matrix tablets and multilayer tablets of metronidazole released 43–52% and 25–44% of the metronidazole, respectively, in the physiological environment of stomach and small intestine depending on the proportion of guar gum used in the formulation. It was observed that both the formulations failed to control the drug release within 5 h of the dissolution study in the physiological environment of stomach and small intestine. The compression-coated formulations released less than 1% of metronidazole in the physiological environment of stomach and small intestine. When the dissolution study was continued in simulated colonic fluids, the compression-coated tablet with 275 mg of guar gum coat released another 61% of metronidazole after degradation by colonic bacteria at the end of 24 h of the dissolution study. The compression-coated tablets with 350 and 435 mg of guar gum coat released about 45 and 20% of metronidazole, respectively, in simulated colonic fluids, indicating the susceptibility of the guar gum formulations to the rat caecal contents. The results of the study showed that compression-coated metronidazole tablets with either 275 or 350 mg of guar gum coat is most likely to provide targeting of metronidazole for local action in the colon owing to its minimal release of the drug in the first 5 h.

Sinha *et al.* [14] designed a formulation with a considerably reduced coat weight and gum concentration for colonic delivery of 5-fluorouracil for the treatment of colorectal cancer. In this study, rapidly disintegrating 5-fluorouracil-loaded tablets coated with 175 mg of granules containing a mixture of xanthan gum and guar gum in varying proportions was prepared. With this coat weight, a highly retarded drug release was observed. After 24 h of dissolution the mean percent drug release from the compression-coated xanthan gum:guar gum 20:20, 20:10 and 10:20 tablets were found to be around 18 ± 1.23%, 20 ± 1.54% and 30 ± 1.77%, respectively. To study the effect of coat weight on drug release profile, the coat weight on the tablets was further reduced to 150 mg. It was observed that reduction of coat weight did not affect the initial drug release rate in simulated upper GIT conditions. At the end of 24 h of dissolution the amount of drug released increased to 25 ± 1.22%, 36.6 ± 1.89% and 42.6 ± 2.22%, respectively, in xanthan gum:guar gum 20:20, 20:10 and 10:20 tablets. Studies of xanthan gum:guar gum (10:20) tablets in presence of colonic contents showed an increased cumulative percent drug release of 67.2 ± 5.23% in presence of 2% cecal content and 80.34 ± 3.89% in presence of 4% cecal content after 19 h of incubation.

Intravenous administration of 5-fluorouracil for colon cancer therapy could produce severe systemic side-effects due to its cytotoxic effect on normal cells. To avoid such problems, recently guar gum tablet formulations were developed for site-specific delivery of 5-fluorouracil to the colon without the drug being released in the stomach or small intestine [15]. In this study, fast disintegrating 5-fluorouracil core tablets were compression-coated with 60%, 70% and 80% of guar gum, and were subjected to *in-vitro* drug release studies. The amount of 5-fluorouracil released from the compression-coated

tablets in the dissolution medium at different time intervals was estimated by a HPLC method. Guar gum compression-coated tablets released only 2.5–4% of the 5-fluorouracil in simulated GI fluids. When the dissolution study was continued in simulated colonic fluids (4% w/v rat caecal content medium) the compression coated with 60%, 70% and 80% guar gum tablets released another 70, 55 and 41% of the 5-fluorouracil respectively. The results of the study show that compression-coated tablets containing 80% of guar gum are most likely to provide targeting of 5-fluorouracil for local action in the colon, since they released only 2.38% of the drug in the physiological environment of the stomach and small intestine. Recently, Murali Kishan [16] developed colon targeting Betulinic acid tablets from guar gum, xanthan gum and tamarind gum using compression coating method. Pre-formulation, weight variation, content uniformity and *in-vitro* dissolution studies were performed using USP apparatus II. Results showed that the guar gum in combination with xanthan gum and tamarind gum showed better drug release profile than when guar gum alone was used.

The crosslinked hydrogels are gaining much importance in a wide variety of applications as super absorbents in wound dressings, as drug carriers, as artificial organs, etc. It was shown that when guar gum is crosslinked with borax, a decrease in viscosity is observed in the presence of enzymes, suggesting that guar gum retains its degradation properties even after crosslinking [17]. However, the borax crosslinked guar gum was not very successful due to its high swelling in the presence of gastric and intestinal fluids. In another study, methotrexate-loaded guar gum microspheres were prepared by the emulsification method using glutaraldehyde as a crosslinking agent for colon-specific drug delivery [18]. It was found that particle size, shape, and surface morphology were significantly affected by guar gum concentration, glutaraldehyde concentration, emulsifier concentration (Span 80), stirring rate, stirring time, and operating temperature. Methotrexate-loaded microspheres demonstrated high entrapment efficiency (75.7%). The *in-vitro* drug release was investigated using a US Pharmacopeia paddle type (type II) dissolution rate test apparatus in different media such as phosphate-buffered saline (PBS), gastrointestinal fluid of different pH and rat cecal content release medium, which was found to be affected by a change to the guar gum concentration and glutaraldehyde concentration. The drug release in PBS (pH 7.4) and simulated gastric fluids followed a similar pattern and had a similar release rate, while a significant increase in percent of cumulative drug release (91.0%) was observed in the medium containing rat cecal content. In *in-vivo* studies, guar gum microspheres delivered most of their drug load (79.0%) to the colon, whereas plain drug suspensions could deliver only 23% of their total dose to the target site.

Phosphated crosslinked low swelling guar gum hydrogels were prepared as shown in Figure 13.2 and analyzed *in vitro* and *in vivo* for their potential as colon drug carriers [19,20]. These hydrogels were loaded with hydrocortisone and were able to resist the release of 80% of the drug for 6 h in phosphate buffer pH 6.4. Addition of α-galactosidase and β-mannanase (enzymes which act upon guar gum) in the buffer solution increased the drug release. *In-vivo* studies in rat showed that modified guar gum was degraded by enzymes in a concentration-dependent manner, showing the suitability of the phosphated crosslinked guar gum for colon drug delivery. In a similar approach, Rubinstein *et al.* [21] have also reported guar gum crosslinked with glutaraldehyde for applications in colon targeting.

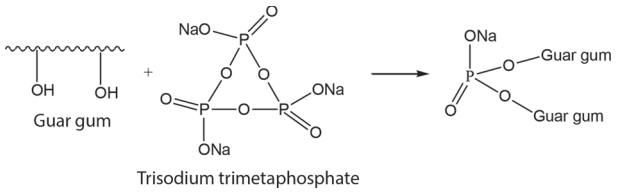

Figure 13.2 Preparation of phosphated crosslinked guar gum.

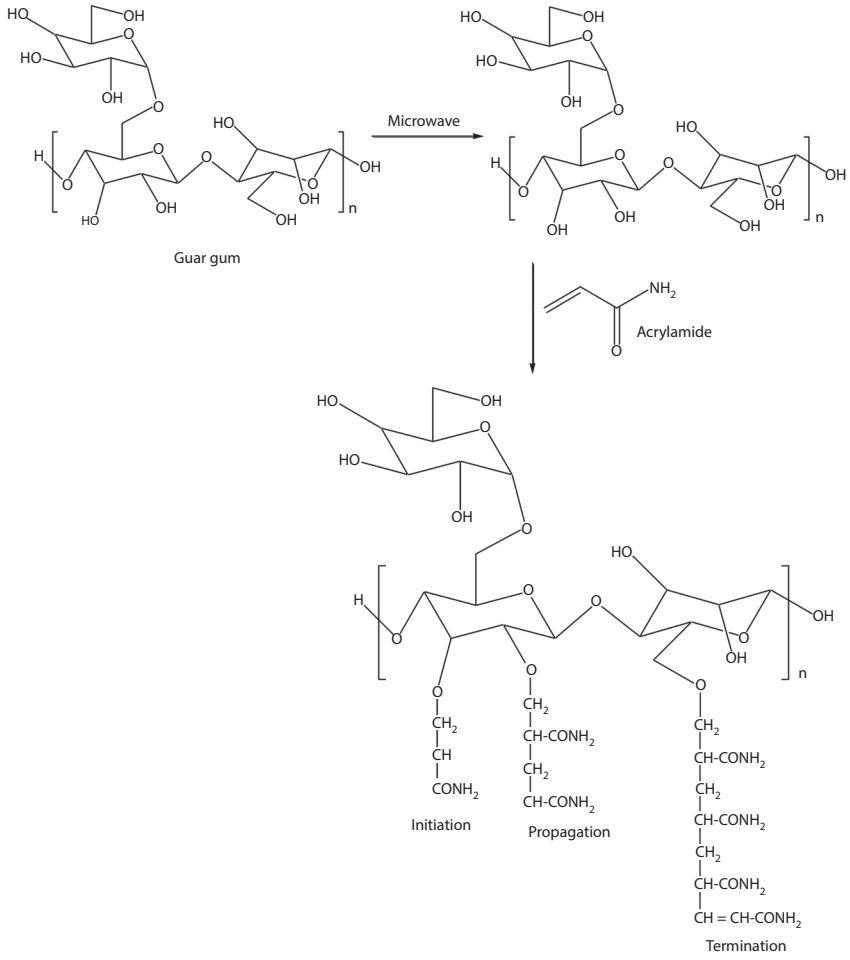

Figure 13.3 Schematic representation for the synthesis of guar gum-*graft*-poly(acrylamide).

Since a major restriction in the design of guar gum matrices for drug delivery is its high swelling characteristics, a chemical modification of guar gum to reduce its enormous swelling properties is a practical alternative solution, especially for orally administered colon-specific drug delivery systems. Recently, guar gum-*graft*-poly(acrylamide) was synthesized as matrix for controlled release of 5-amino salicylic acid, as shown in Figure 13.3 [22]. The applicability of this matrix for sustained drug release has been

investigated by the United States Pharmacopeia (USP) drug dissolution method, under different pH environments. It has been found that the higher the percentage of grafting, the lower is the rate of drug release. Further, it has been observed that the rate of release of the enclosed drug from grafted guar gum matrix is low in acidic environment and is higher in neutral and alkaline environment, thus raising the possibility of further optimization of grafted guar gum matrix as a potential candidate for lower GIT-targeted drug delivery.

Cationic guar gum/poly(acrylic acid) polyelectrolyte hydrogels was prepared through photoinitiated free radical solution polymerization as colon-specific drug delivery carrier [23]. In this work, the cationic guar gum was prepared by reacting guar gum with 3-chloro-2-hydroxypropyltrimethylammonium chloride in presence of sodium hydroxide, as shown in Figure 13.4. The release studies showed that the compositions of the hydrogel had an important effect on ketoprofen release. The increase of poly(acrylic acid) content conduced the rapid release of ketoprofen from the polyelectrolyte hydrogels. It was found that the ketoprofen release followed non-Fickian mechanism. Furthermore, it was observed that polymer erosion was a dominating factor in the release process of the tablet prepared by compression. The pH of the dissolution medium appeared to have a strong effect on the drug transport mechanism. At more basic pH values, a drug release mechanism was highly influenced by macromolecular chain relaxation. In this study, the ketoprofen release was also tested under the conditions chosen to simulate the pH and time interval likely to be encountered during transit from stomach to colon. The results implied that the polyelectrolyte hydrogels can be exploited as potential carriers for colon-specific drug delivery.

13.2.1.2 Antihypertensive DDSs

The use of guar gum as controlled release matrix for antihypertensive drugs such as ketoprofen, nifedipine and diltiazem hydrochloride has been reported [24,25]. These studies reported that dissolution of hydrophilic drugs from guar gum formulations is nearly independent of stir speed under normal dissolution conditions. The prepared formulations in these studies provided prolonged drug release under both *in-vitro* and *in-vivo* conditions. In another study, matrix tablets of diltiazem hydrochloride, using various viscosity grades of guar gum in two proportions, were

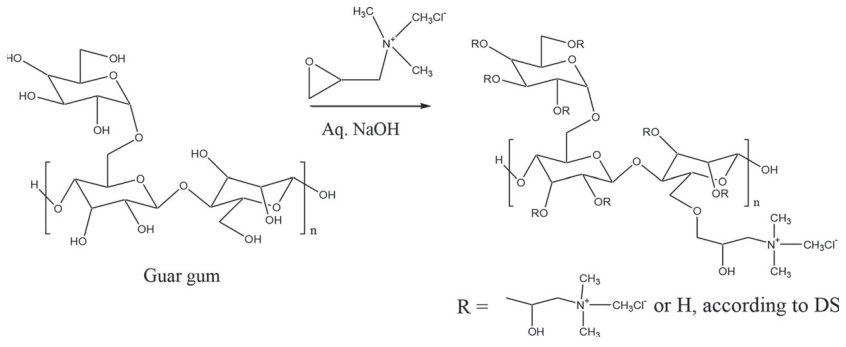

Figure 13.4 Synthesis of cationic guar gum.

prepared by wet granulation method for oral controlled release [26]. Diltiazem hydrochloride matrix tablets containing either 30% w/w low viscosity, 40% w/w medium viscosity or 50% w/w high viscosity guar gum showed controlled release. The drug release from all the guar gum matrix tablets followed first-order kinetics via Fickian-diffusion mechanism. Further, the results of *in-vitro* drug release studies in simulated gastrointestinal and colonic fluids showed that high viscosity tablets provided controlled release comparable with commercial diltiazem hydrochloride tablets. When subjected to *in-vivo* pharmacokinetic evaluation in healthy volunteers, the high viscosity tablets provided a slow and prolonged drug release when compared to commercial tablets. Based on the results of *in-vitro* and *in-vivo* studies it was concluded that guar gum matrix tablets provided oral controlled release of diltiazem hydrochloride drug.

Oral controlled delivery systems for antihypertensive drug trimetazidine dihydrochloride using guar gum as a carrier were also reported [27]. In this study, three-layer matrix tablets with 200 mg of release retardant layer containing either 65%, 75% or 85% of guar gum over matrix formulation containing various proportions of guar gum were prepared. The three-layer matrix tablet with 200 mg of 85% of guar gum layers over matrix formulation containing 50% of guar gum was found to provide the required release rate matching with the theoretical release rates. Based on dynamic scanning calorimetric (DSC) studies, it was found that there is no possible interaction between trimetazidine dihydrochloride and guar gum/other excipients used in the matrix tablets. The results clearly indicate that guar gum in the form of a three-layer matrix system is a potential hydrophilic carrier in the design of oral controlled drug delivery systems for highly soluble drugs.

Stimuli-responsive nano/microgels can respond to external stimuli such as pH, ionic strength, temperature, and electric current. Such polymeric systems are useful as stimulus responsive drug carriers for several classes of drugs. Soppirnath and Aminabhavi [28] have reported the glutaraldehyde crosslinked poly(acrylamide)-*graft*-guar gum hydrogel microspheres for the controlled release of calcium channel blockers like verapamil hydrochloride and nifedipine (Figure 13.5). In these studies, the drugs were incorporated either during crosslinking by dissolving them in the reaction medium or after crosslinking by the soaking technique. Dynamic swelling experiments indicated that with an increase in crosslinking, water transport deviates from Fickian to non-Fickian mechanism. The *in-vitro* drug release showed a dependence on the extent of crosslinking, amount of drug loading, nature of drug molecule and method of drug loading. The hydrogel microspheres showed a swelling followed by diffusion controlled drug release mechanism.

Toti and Aminabhavi [29] have developed poly(acrylamide)-*graft*-guar gum and hydrolyzed poly(acrylamide)-*graft*-guar gum as the matrix tablet for the controlled release of diltiazem hydrochloride. For poly(acrylamide)-*graft*-guar gum matrix, the release was found to be continued up to 8 h. In the case of hydrolyzed poly(acrylamide)-*graft*-guar gum matrix, release time increased with increasing grafting ratio of the grafted copolymer, which continued up to 12 h. Hydrolyzed poly(acrylamide)-*graft*-guar gum matrices released only 27% of the total drug in gastric pH, while the rest of the drug was released in the intestinal pH conditions. Hence, the systems of this study can be good candidates for intestinal drug delivery. In another study, lipase-functionalized guar gum

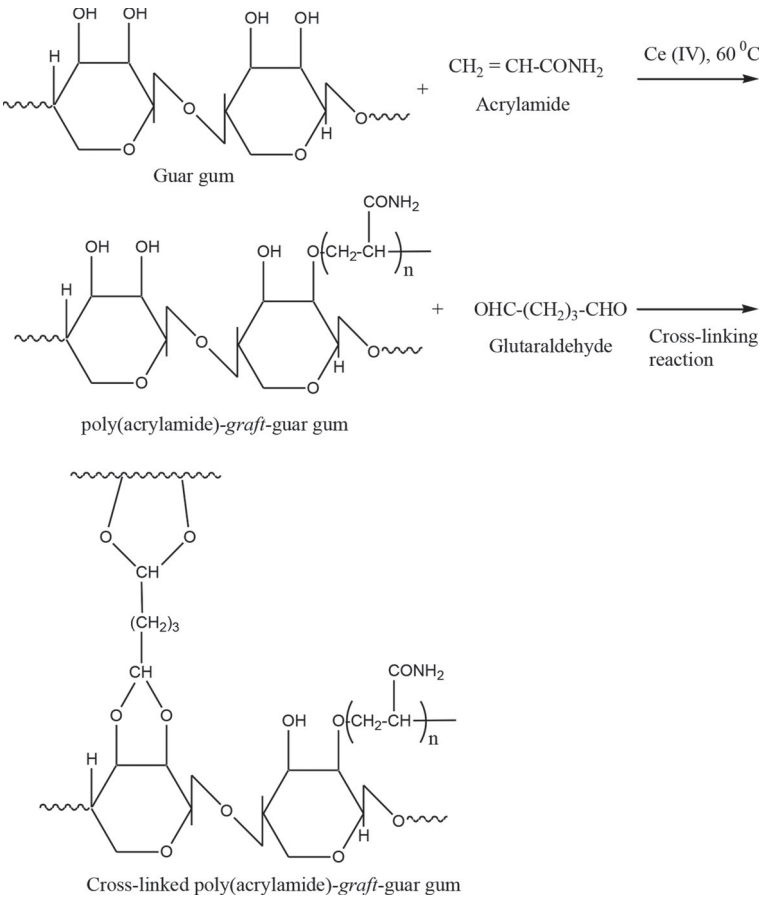

Figure 13.5 Formation of crosslinked poly(acrylamide)-*graft*-guar gum.

nanoparticles in the size range of 19–32 nm were prepared by nanoprecipitation and crosslinking method for drug delivery applications [30]. In this work, the efficacy of the drug release on the guar gum nanocarrier was demonstrated indirectly by the release of crystal violet. The release kinetics indicated that the release was faster till 24 h, and thereafter the release was very slow. This result suggests that guar gum-based nanosized materials could be potentially used as drug delivery carriers.

13.2.1.3 Transdermal DDSs

A transdermal drug delivery device, which may be of an active or a passive design, is a device which provides an alternative route for administering medication. These devices allow for pharmaceuticals to be delivered across the skin barrier [31]. A drug is applied in a relatively high dosage to the inside of a patch, which is worn on the skin for an extended period of time. Through a diffusion process, the drug enters the bloodstream directly through the skin. Since there is high concentration on the patch and low concentration in the blood, the drug will keep diffusing into the blood for a long period of time, maintaining the constant concentration of drug in the blood flow.

Carboxymethyl guar gum was evaluated for its suitability of use in transdermal drug delivery systems using terbutaline sulfate as a model drug [32]. The polymer exhibited good film-forming ability and therefore used to prepare films possessing desired properties by varying the composition of the casting solution. The release study showed that the diffusion of terbutaline sulfate from carboxymethyl guar gum solution was relatively slower at pH 5 than at pH 10. This observation might be due to the static interaction between carboxymethyl guar gum and terbutaline sulfate at pH 5. It was observed that the ionized/unionized state of drug is an important factor to be considered while preparing the casting solution to induce or minimize the interaction between the polymer and the drug.

Thakur *et al.* [33] developed acryloyl guar gum hydrogels as transdermal drug delivery devices. In this study, L-tyrosine and 3,4-dihydroxy phenylalanine (L-DOPA) were used as the model pro-drugs. L-tyrosine is involved in the synthesis of neurotransmitters in the brain. It is a precursor to L-DOPA, nor-epinephrine and epinephrine. The results showed that these hydrogel materials exhibited unique swelling behavior, and responded well to the physiological stimuli such as pH and the ionic strength. The high loading of L-tyrosine and L-DOPA was achieved on these hydrogel materials. Release studies showed that the release behavior of these hydrogels was slow, especially in the medium of pH 7.4. The hydrogel materials exhibited structure-property relationship in the release of both L-tyrosine and L-DOPA. The % cumulative release of L-tyrosine was found to be maximum from the acryloyl guar gum containing poly(methacrylic acid), while the maximum release of L-DOPA was observed from acryloyl guar gum containing poly(acrylic acid) in both the media. On the other hand, acryloyl guar gum based on 2-hydroxyethyl methacrylate and 2-hydroxypropyl methacrylate showed a sustained release of both L-tyrosine and L-DOPA even after 12 h. These results indicate that acryloyl guar gum hydrogels could be used as pro-drug delivery carriers for transdermal applications.

A hydrophobic device for controlled transdermal release of diclofenac sodium was designed from the nanosilica/acrylic acid grafted guar gum membranes [34]. Nanocomposite/drug conjugates were formed by bringing down the medium pH from 9.0 to 7.0. The graft-copolymer nanocomposites provided excellent control over diclofenac release due to high hydrophobicity and better cage morphology. All nanocomposite conjugates studied in this work had limited biocompatibility from which better skin compatibility and greater membrane life could be anticipated. Recently, Bhunia *et al.* [35] prepared a transdermal device from 2-hydroxyethyl methacrylate grafted carboxymethyl guar gum-functionalized multi-walled carbon nanotube (MWCNT) *in-situ* composite membranes for sustained delivery of diclofenac sodium. Polymer matrix-MWCNT interaction at 0.5 and 1 wt% MWCNT concentrations induces excellent copolymer-adsorbed fibrillar orientation of MWCNT compared to that at 2 and 3 wt%. It successively leads to efficient encapsulation and more sustained release of the drug molecules. Results showed that the releases are dominated by the viscoelastic relaxation behavior of the devices. Conversely, for the latter, the releases are comparatively less sustained and more swelling controlled. However, all devices have significantly increased the half-life period of the drug molecules.

13.2.2 Tissue Engineering Scaffolds

The field of tissue engineering holds great promise for the repair or regeneration of damaged and diseased tissues. Its underlying objective is to direct a population of cells into forming a living tissue, structurally and functionally indistinguishable from that found in nature. To guide the transition from cells to tissue, a three-dimensional scaffold will be utilized [36]. Together with the choice of appropriate cells and bioactive agents, the suitable material for the scaffold preparation plays a crucial role for the success of the application. In this context, considerable attention has been given to polysaccharides because of their desirable properties for biomedical applications.

Although a number of hydrogels have been explored as scaffolding materials for cartilage, corneal and heart valve tissue engineering applications using a range of ionic polysaccharides, such as chitosan, alginate, hyaluronic acid and other glycosaminoglycans [37–40], certain limitations exist with ionic hydrogels due to the nature of the ionizable groups [41]. The dynamic swelling equilibrium of ionic hydrogels is closely related to pH, ionic strength, temperature and composition of the external solutions. Since ionic hydrogels are pH responsive, they may form biologically stable ionic networks due to the strong ionic interaction. A high degree of ionic crosslinking in ionic gels is likely to significantly reduce the network mesh size and the degree of swelling. To overcome these issues, recently guar gum-based nonionic polysaccharides have been considered for tissue engineering scaffold applications. Zhao *et al.* [42] prepared semi-interpenetrating network (semi-IPN) hydrogels based on poly(ethylene glycol)-co-poly(ε-caprolactone) diacrylate macromer and hydroxypropyl guar gum by a low intensity ultraviolet (UV) light irradiation method, and characterized by FT-IR, DSC and WAXD analysis. Their properties were evaluated by investigating the swelling kinetics, dynamic mechanical rheology and the release behavior for bovine serum albumin (BSA). It was found that the introduction of the semi-IPN structure and hydroxypropyl guar gum decreased the crystallinity of poly(ethylene glycol) segments in the hydrogel, and improved the swelling and mechanical properties of the hydrogel, as well as lowered the release percentage of BSA from the hydrogel. The authors suggested that semi-IPN hydrogels based on poly(ethylene glycol)-co-poly(ε-caprolactone) diacrylate macromer and hydroxypropyl guar gum may have more advantages as a potentially interesting platform for the design of tissue engineering scaffolds.

Tiwari *et al.* [5] fabricated guar gummethacrylate hydrogel as tissue engineering scaffolds by photopolymerization of water-soluble guar gum methacrylate macromonomers, as shown in Figure 13.6. The prepared guar gum–methacrylate hydrogels exhibited a 3-dimensional, open cell microstructure with an average pore size ranging from 10 to 55 μm. The hydrogels exhibited equilibrium swelling ratios ranging from ~22% to 63%. The degree of *in-vitro* enzymatic biodegradation of the hydrogels decreased linearly with increasing gel content and the degree of methacrylation of the respective macromonomers. Cell viability and cell proliferation were tested with a human endothelial cell line, EA.hy926, to explore the potential use of guar gum–methacrylate hydrogels for *in-situ* tissue engineering scaffold applications, which showed promising results. Guar gum–methacrylate hydrogels with a 0.05 wt% macromonomer concentration revealed excellent endothelial cell proliferation, similar to that of the Matrigel™ control.

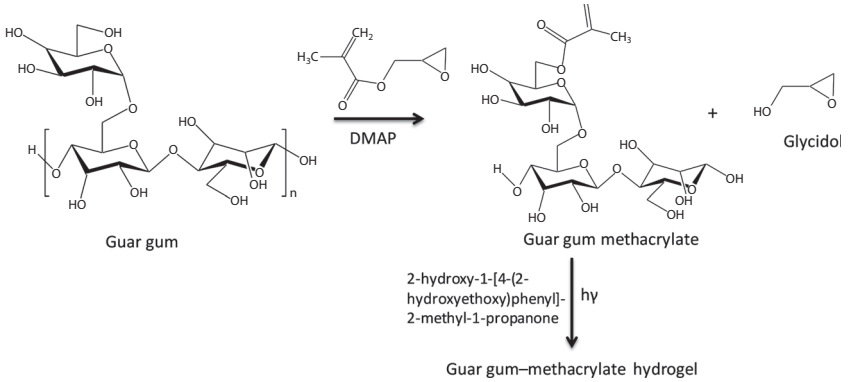

Figure 13.6 Synthesis of guar gum–methacrylate hydrogel.

In recent years, collagen-based biomaterials are being developed for a number of tissue engineering and medical applications [43]. The attractiveness of collagen as a biomaterial rests largely in the view that it is a natural material of low immunogenicity and is therefore seen by the body as a normal constituent rather than foreign matter. However, thermal stability of collagen is not sufficient for many in-vivo and in-vitro applications. In order to render collagen suitable for tissue engineering applications, the mechanical strength of collagen must also be enhanced, which can be achieved through blending and/or crosslinking with biodegradable polysaccharides [44]. Recently, Manikoth *et al.* [45] studied the effect of guar gum on the thermal stability, dielectric property and pore size distribution of collagen-based biocomposite. It was observed that guar gum causes changes in the rheological properties of the collagen with no effect on the native protein structure. Moreover, the different concentration of guar gum resulted in four distinct composites with different mean pore diameters and distribution. Each composite had a consistent pore structure and showed no obvious variation in mean pore size, structure, or alignment at separate points within the composites, indicating the homogeneity of composites produced. The pore size distribution of the biocomposite matrices can be shifted to the lower nanometer range by varying the guar gum concentration. Homogeneity of the pore sizes was increased with the increase of guar gum concentration. Composites with smaller pores have a greater surface area which influences ligand–integrin interaction, facilitating the initial cellular attachment. Changes in functional group and morphology of composites indicate that collagen interacts with guar gum chemically and physically. Increase in concentration of guar gum results in increase in thermal stability. The polarizability of the collagen is tuned by the guar gum concentration. Ionic charge drift creates conduction currents and initiates polarization mechanisms through charge accumulation at structural interfaces. Their dielectric properties reflect contributions to the polarization from both structure and composition of the designed biomaterial. Behavioral changes of the rotational as well as vibrational motion of the polar functional groups associated with the protein are due to the formation of hydrogen bond between functional groups of protein and guar gum. This leads to the alteration of the hydration shell of the collagen. This study highlights that composites with variable pore sizes optimal for cell growth can be achieved by varying the guar gum concentration, which can be used for tissue engineering applications.

13.2.3 Wound Healing Materials

Wound healing is an innate physiological response that helps restore cellular and anatomic continuity of a tissue. An ideal dressing should maintain a moist environment at the wound interface, allow gaseous exchange, act as a barrier to microorganisms and remove excess exudates. It should also be nontoxic, non-allergenic, non-adherent and easily removed without trauma, it should be made from a readily available biomaterial that requires minimal processing, possesses antimicrobial properties and promotes wound healing [46]. In this regard, while selective biodegradable and biocompatible polymer materials such as collagen, alginic acid, chitin, chitosan, hyaluronic acid, etc., have been reported as useful scaffolds for wound healing and assisted cellular messaging, only limited information has been reported on guar gum-based wound healing materials. Auddy *et al.* [47] reported a chemically modified guar gum derivative, cationic biopolymer guar gum alkylamine, for wound healing applications. In this work, biologically synthesized silver nanoparticles were further impregnated in guar gum alkylamine for extended evaluations in punch wound models in rodents. SEM studies showed that silver nanoparticles were well dispersed in the new guar matrix with a particle size of 18 nm. In wound healing experiments, faster healing and improved cosmetic appearance were observed in the new nanobiomaterial treated group compared to commercially available silver alginate cream. The total protein, DNA, and hydroxyproline contents of the wound tissues were also significantly higher in the treated group as compared with the silver alginate cream. Silver nanoparticles exerted positive effects because of their antimicrobial properties. The nanobiomaterial was observed to promote wound closure by inducing proliferation and migration of the keratinocytes at the wound site. It is confirmed that the derivatized guar gum matrix additionally provided a hydrated surface necessary for cell proliferation.

Recently, Sevinc *et al.* [48] investigated the effects of preoperative oral administration of partially hydrolyzed guar gum (Benefiber®) on the healing of irradiated colonic anastomosis. In this study, forty male Wistar rats were divided into four groups. Group I (control group), Group II (Benefiber® pretreatment group), Group III (preoperative radiotherapy group) and Group IV (preoperative radiotherapy and Benefiber® pretreatment group). All animals underwent 1cm left colon resection and primary anastomosis. On the 3rd and 7th postoperative days, all the rats were anesthetized to assess the anastomotic healing clinically, mechanically, histologically and biochemically. The results showed that the mean bursting pressure was significantly lower in group III and significantly higher in group II on day 7. The histologic parameters of anastomotic healing, such as epithelial regeneration and formation of granulation tissue, were significantly improved by use of preoperative Benefiber® on day 7. The amount of acid-soluble collagen concentrations significantly increased in group IV compared to group III on day 3. The amount of salt-soluble collagen concentrations significantly increased in group II compared to group III on day 3. These results conclude that colonic anastomotic healing can be adversely affected by preoperative radiotherapy, but orogastric feeding with Benefiber may improve the healing process.

13.2.4 Biosensors

A biosensor is a device which intimately couples a specific biological recognition element with a physical transducer, such as amperometric, potentiometric, optical, etc.,

and translates the biological recognition event into a corresponding physical signal. Different types of biosensors based on biopolymers have been developed for the detection of biomolecules in the complex environment using enzyme immobilized electrodes [49]. The performance of such biosensors usually depends on the stability and activity of the enzyme immobilized on the electrode surface. In order to improve the stability and activity of the enzyme immobilized on the electrode surface, a variety of methods have been tried such as adsorption, entrapment in a porous matrix, covalent binding and electrochemical copolymerization. Immobilization of enzyme may lead to changes in enzyme structure and hence causes the kinetics, stability and specificity to differ from that of the enzyme in homogeneous solution [50]. Therefore, great interest is focused on finding immobilization matrix, which can retain its specific biological function. In recent years, there has been a significant increase of interest in using guar gum and its derivatives as an immobilization matrix for enzymes, living organisms, and cell carriers.

Bagal and Karve [6] immobilized plant invertase enzyme in the composite of agarose–guar gum biopolymer matrix in the form of hydrophilic, porous membranes. Agarose–guar gum composite has a number of advantages as a matrix for enzyme immobilization. Both are nontoxic and hydrophilic in nature, providing a natural environment for the entrapped enzyme. In this study, the immobilized invertase was characterized for sucrose hydrolytic activity and leakage from the matrix support. The efficiency of immobilization was found to be 91% with negligible leaching. Immobilized invertase was optimally active in the wide pH range of 4.5–6.5. The immobilization process also enhanced the thermal stability of enzyme up to 65°C. Entrapped invertase showed better operational stability and reusability up to 12 cycles. The fluorescence spectroscopic studies suggested that the agarose–guar gum composite provides the conformational stability to invertase without any modification. The storage stability for agarose–guar gum composite matrix was found to be high as compared to other biopolymers. These results suggest that the nontoxic and biocompatible agarose–guar gum biopolymer matrix can gain prime importance in the fabrication of biosensors employed in the food industry. Tembe *et al.* immobilized tyrosinase from a plant source, *A. companulatus,* in the agarose–guar gum composite matrix [51,52]. This composite matrix-containing enzyme forms a self-adhering layer on the active surface of glassy carbon electrode, making it a selective and sensitive phenol sensor. Dopa and dopamine were determined by the direct reduction of biocatalytically liberated quinone species at -0.18V versus Ag/AgCl (3M KCl). The sensor performance was characterized in terms of sensitivity, linearity, detection limit, pH, and storage stability. The results showed that this simple, easy to fabricate, reagentless electrode is suitable for quantification of catecholamine at the micromolar level.

An evanescent-wave biosensor was designed by using agarose–guar gum biopolymer composite matrix with entrapped enzymes (acid invertase and glucose oxidase) for the fabrication of sucrose biosensor, as shown in Figure 13.7 [53]. The composite matrix of agarose–guar gum forms a self-adhesive membrane on the surface of optical planar waveguide. In this study, the optogeometric properties of acid invertase and glucose oxidase entrapped cladding layer at the surface of waveguide sensor were studied. The response of biosensor was found to be linear in a wide range of sucrose

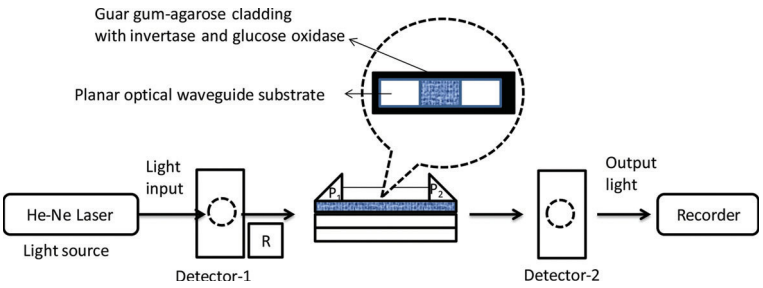

Figure 13.7 Schematic representation of invertase-glucose oxidase entrapped guar gum-agarose-based biosensor.

concentration. Maximum sensitivity for sensor was observed over wide acidic pH with fast response. The results revealed that the optical waveguide biosensor has a very high sensitivity with lower detection limit of 2.5×10^{-11}M for sucrose. The fabricated biosensor showed good operational and storage stability. It was observed that the planar optical waveguide sensor is versatile, easy to fabricate and can be used for the measurements of different analytes such as glucose, urea, etc., using glucose oxidase and urease as biomaterial, respectively.

An electrochemical sucrose biosensor using ultramicroelectrode has been reported for the detection of heavy metal ions [54]. In this work, the working UME was modified with invertase and glucose oxidase entrapped in agarose–guar gum. The atomic force microscopy (AFM) images of the membranes showed proper confinement of both the enzymes during co-immobilization. The dynamic range for sucrose biosensor was achieved in the range of 1×10^{-10} to 1×10^{-7} M, with lower detection limit 1×10^{-10}M at pH 5.5 with 9 cycles of reuse. The dynamic linear range for mercury using electrochemical biosensor was observed in the range of 5×10^{-10} to 12.5×10^{-10} M for sucrose. The lower detection limit for the fabricated biosensor was found to be 5×10^{-10} M. The kinetic parameters of inhibition by metal ions were studied by 3,5-dinitrosalicylic acid method. A higher sensitivity was achieved amperometrically by employing platinum ultra-microelectrode instead of disc Pt electrode. The experimental results proved the enhanced analytical performance of the biosensing system in order to estimate sucrose and metal ions. The results suggest that the enzyme-modified ultramicroelectrode-based sucrose sensor is a promising tool for the estimation of heavy metal ions and real on-site sample analysis. Recently, Pandey *et al.* prepared guar gum/silver nanoparticles nanocomposite as an ammonia sensor [55]. In this study, the ammonia sensing property of guar gum/silver nanoparticles nanocomposite was performed by optical method based on surface plasmon resonance (SPR). The response time of 2–3 s and the detection limit of ammonia solution, 1 ppm were found at room temperature. This result suggests that the guar gum/silver nanoparticles nanocomposite-based ammonia sensor can be used for clinical and medical diagnosis for detecting low ammonia level in biological fluids, such as plasma, sweat, saliva, cerebrospinal liquid or biological samples in general for various biomedical applications in humans.

13.2.5 Antimicrobial Agents

A number of chemicals have been used as antimicrobial agents. Those chemicals include inorganic salts, organometallics, iodophors, phenols and thiophenols, onium salts, antibiotics, heterocyclics with anionic groups, nitro compounds, ureas and related compounds, formaldehyde derivatives, and amines [56]. Many of these chemicals, however, are toxic to humans and do not easily degrade in the environment. These facts have facilitated the use of biodegradable natural polymers as novel antimicrobial agents for application in textile, food and water technology. In recent years, hydrophobic modifications on guar gum was considered interesting and can extend a significant possibility for application in different areas like water technology, pollutant removal, bioplastics and biomedical devices. Different functionalized guar gum derivatives were explored relatively recently as antimicrobial materials.

Das *et al.* [57] developed a water-resistant biocide film based on guar gum for applications in food and water technology. In this work, guar gum was intrinsically modified to a new guar gum benzamide. Benzoylation was carried out by benzoyl chloride reaction in water medium and a propyl amine spacer was used to impart a high degree of hydrophobicity. The guar gum benzamide was found to be resistant to water and soluble in nonaqueous solvent like dimethyl sulfoxide. Cast films of thickness 0.162 mm had a breaking point tensile strength of 21.95 MPa. The water vapor permeability of biomaterial film was 0.28 g mm $kPa^{-1}h^{-1}m^{-2}$ and water contact angle on evaporative surface was 90.35°. Qualitative and quantitative biocide activity of film was established against *Salmonella enterica*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. These results showed that guar gum benzamide had good antimicrobial activity against these microornanisms.

Guar gum and chitosan composite films were prepared by the casting method using guar gum and chitosan in different ratios [58]. The concentration of guar gum ranged from 0% to 50% (v/v). The optical properties such as transparency, opacity and color were measured. Water vapor transmission rate and oxygen permeability of the films were also investigated. Films were evaluated for mechanical and antibacterial properties. Addition of guar gum in varied proportions to chitosan solution led to changes in transparency and opacity of films. The water vapor transmission rate did not change significantly upon addition of guar gum. Films containing 15% (v/v) guar gum showed very low oxygen permeability, and good tensile and puncture strength. The antimicrobial activity of films containing 15% (v/v) guar gum was comparable to chitosan films against *Escherichia coli* and *Staphylococcus aureus*. The authors suggested that composite films obtained from guar gum and chitosan may reduce environmental problems associated with synthetic packaging. Recently, Das and Mukherjee reported a hydrophobically modified guar gum film for soluble pollutant sorption and reduction of microbial load in water environment [59]. This modified guar gum derivative was found to be insoluble in water and formed castable films with a high degree of surface hydrophobicity. The film water vapor permeability was 0.233 ± 0.029 g mm kPa^{-1} h^{-1} m^{-2} and the tensile property was 38.65 ± 3.66 MPa. The film surface contact angle against water was 77.36 ± 1.09°. The hydrophobically modified guar gum film demonstrated strong antimicrobial contact killing against both gram positive and gram negative bacteria. It also acted as an efficient sorbent for a range of water- soluble organic pollutants.

13.3 Conclusions

Guar gum and its derivatives are stable, safe and biodegradable. Due to these favorable properties, they are widely used as biomaterials in pharmaceutical applications such as DDSs, tissue engineering scaffolds, wound healing materials, biosensors and antimicrobial agents. The therapeutic effect of guar gum and its derivatives is due to their ability to swell rapidly in aqueous media to form viscous dispersions or gels. Guar gum and its derivatives are widely used as colon-specific, antihypertensive and transdermal DDSs in the form of matrix and compression-coated tablets as well as microspheres. When mixed with different ingredients in formulation of matrix tablets, they form protective layer and, consequently, drug releases out from the matrix in a sustained manner, achieving the desired kinetics effect. Since guar gum-based hydrogels are biodegradable and exhibited a 3-dimentional, open cell microstructure with an adequate pore size and pore density, they have potential to be used as tissue engineering scaffolds. The composite material based on guar gum and agarose was found to have high permeability towards water, provide natural microenvironment to the enzyme and give sufficient accessibility to electrons to shuttle between the enzyme and the electrode. Therefore, the guar gum–agarose composite material can be used to fabricate an efficient biosensor for the measurements of different analytes such as glucose, urea, etc. Guar gum combined with suitable functional materials have demonstrated strong antimicrobial activity against microorganisms and therefore they can be used for the development of novel wound healing and antimicrobial agents.

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14

Polymers for Peptide/Protein Drug Delivery

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Abstract

A substantial number of protein drugs have been introduced to the pharmaceutical industry due to the intensive research investigating the therapeutic potential of proteins and peptides. However, although proteins and peptides are valuable active pharmaceutical ingredients, they have certain inherent problems such as *in-vivo* instability and a short biological half-life. This is the reason why developing a carrier for the delivery of this kind of biological active represents a great challenge. To solve these problems encapsulation techniques have been developed. Micro- or nanoencapsulation can envelop protein/peptide drugs within a polymeric matrix in order to protect them from degradation and modulate the biological active release. This chapter examines the latest developments in the field of protein and peptide encapsulation using biopolymers as carriers, focusing specifically on polymers derived from lactic acid and other natural biodegradable polymers.

Keywords: Peptides, proteins, biopolymers, microparticles, nanoparticles, drug delivery

14.1 Biodegradable Polymers

Biodegradable polymers can be natural or synthetic in origin and they are degraded *in vivo,* either enzymatically or non-enzymatically or both, producing a biocompatible byproducts which are further eliminated by the normal metabolic pathways.

The number of such materials that are used in or as adjuncts in controlled drug delivery has increased dramatically over the past decade. The basic category of biomaterials used in drug delivery can be classified as shown in Figure 14.1. There are two important categories:

1. Synthetic biodegradable polymers, and
2. Naturally occurring polymers [1–3].

The selection of materials used in drug delivery is based on several factors such as dosage range and special requirements that may apply. In addition, biocompatibility is

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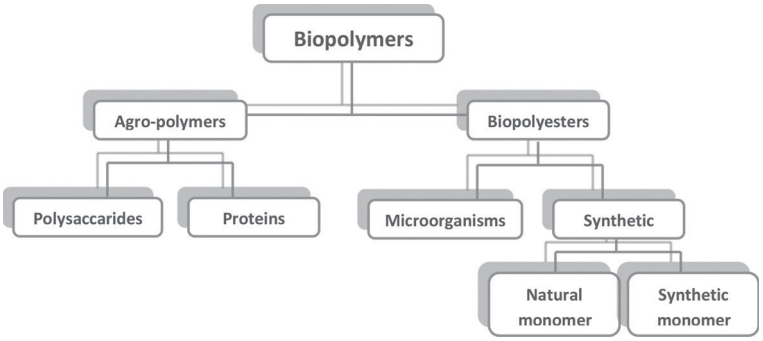

Figure 14.1 Classification of biopolymers.

crucial for this kind of application, but it is not an intrinsic property of a material, but depends on the biological environment and the tolerability to specific drug-polymer-tissue interactions [3].

14.2 Why Protein and Peptide Encapsulation?

A substantial number of protein drugs have been introduced to the pharmaceutical industry due to the intensive research investigating the therapeutic potential of proteins and peptides. Now, various health-endangering diseases, such as cancer, infectious diseases, diabetes and autoimmune diseases, can be treated using protein drugs, including aldesleukin, human growth hormone, bone morphogenic protein, insulin and others [4,5].

However, although proteins and peptides are valuable active pharmaceutical ingredients, they have certain inherent problems summarized in Figure 14.2, such as *in-vivo* instability and a short biological half-life. This is the reason why developing a carrier for the delivery of this kind of biological active represents a great challenge [6].

Protein and peptide drugs are characterized by some unsolved difficulties, such as a short biological half-life time, mainly because they are easily hydrolyzed or degraded by enzymes *in vivo* and the fact that most of them cannot diffuse across some biological barriers [7]. Therefore, frequent injections are usually needed. This goes against the welfare of the patient and also entails economic burdens. Moreover, other administration routes, such as oral and mucosal, are complicated due to their easy degradation character and large molecular size.

There are two main problems in obtaining polymeric micro- and nanoparticles, and these issues represent an obstacle when you want to scale up a product of its kind to be offered in the market.

1. Size and size distribution of the particles are difficult to control, resulting in poor reproducibility in large-scale production; for this reason the drug delivery system may fail to be approved.
2. It is also difficult to maintain the bioactivity of protein/peptide drugs during the preparation, storage, and release of the drug. If these biological actives suffer deactivation the therapeutic effect no longer exists [8–11].

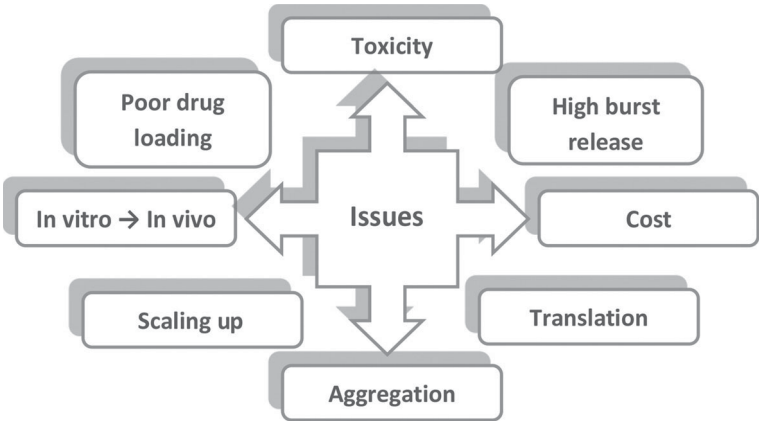

Figure 14.2 Issues for the production and commercialization of biodegradable particles loaded with protein/peptides.

To solve these problems encapsulation techniques have been developed [12,13]. Micro- or nanoencapsulation can envelop protein/peptide drugs within a polymeric matrix in order to protect them from degradation and modulate the biological active release [14].

14.3 Surface Functionalization

The body recognizes hydrophobic particles as foreign. The reticulo-endothelial system (RES) eliminates these from the blood stream and takes them up in the liver or the spleen. *This process is one of the most important biological barriers to particles-based controlled drug delivery* [15].

In order to overcome these limitations, several surface modification methods have been developed to produce particles that are not recognized by the RES. The need for improved delivery formulations that incorporate a variety of drugs and methods of administration has driven the development of several types of block copolymers of polyesters with poly ethylene glycol (PEG) [16–18]. Micro- and nanoparticles can be coated with molecules that cover the hydrophobicity by, for example, giving a hydrophilic layer at the surface. The most extensively used surface modification is "PEGylation," which has been largely demonstrated to increase the blood circulation half-life by several orders of magnitude [19]. In addition, surface modification produces the targeting of tumors, increasing the selective cellular binding and internalization through receptor-mediated endocytosis [20]. It is also known that the surface charges of nanoparticles have an important influence on their interaction with cells and on their uptake.

Nevertheless, it has been proved that the addition of PEG to the system produces a reduction of encapsulation efficiency for drugs and proteins, even when the appropriate fabrication technique is used. That reduced drug inclusion could be related to steric interference of drug/protein-polymer interaction by the PEG chains, but until now, the precise mechanism for this effect was not elucidated. Improved release kinetics

of formulations of PLGA-PEG copolymers have been demonstrated in comparison to PLGA alone, whereas several mechanisms of targeted delivery of drugs from PLGA-PEG micro- and nanoparticles have also been reported [17,21,22].

By definition, opsonization is the process by which an alien particle or organism is covered with opsonin proteins, promoting aggregation and highlighting it for macrophages. After this, phagocytic cells engulf and dispose of these strange materials from the bloodstream (Figure 14.3). The synergy of these two processes results in the main clearance mechanism for the removal of undesirable agents in the human organism [19,23–26]. Most of the opsonized particles are cleared by a receptor-mediated mechanism [27].

In order to improve the blood circulation half- life of particulate protein and peptide carriers based on the biopolymers mentioned above, several methods of camouflaging or masking them from the reticuloendothelial system (RES) have been developed [28,29]. Surface functionalization can overcome the most important limiting factor for long-circulating carriers: protein absorption. In accordance with this, the most relevant strategy to interfere with the binding of opsonin proteins and consequently avoid the mononuclear phagocytic system, is making use of surface treatments [19]. The main objective is to increase blood residence and accumulation in the adequate tissues for the treatment of a specific disease; numerous approaches to reach this goal have been

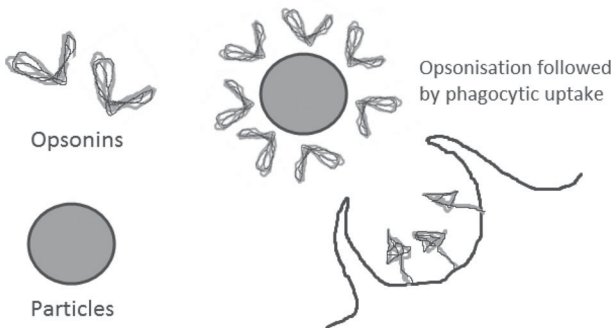

Figure 14.3 Scheme of particles phagocytosis as a result of the opsonins surface attachment.

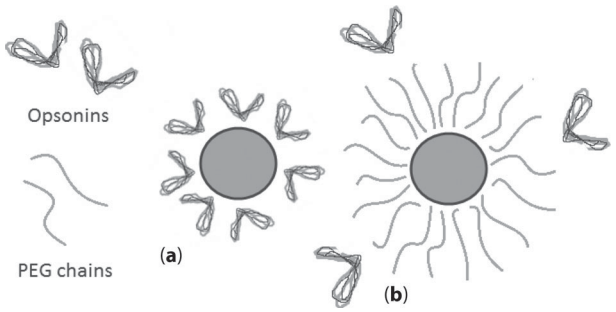

Figure 14.4 a) Particle is marked by opsonins and b) PEG-coated micro/nanoparticle avoid opsonins recognition.

developed. It is well known that hydrophilic polymers, especially PEG, can be associated to the surface of particulate drug delivery systems and provide them steric stabilization and "stealth" properties such as prevention of protein absorption (Figure 14.4) [18]. PEG-containing surface treatments for nanodrug containers seem to represent one of the most promising strategies and show the lowest occurrence of harmful effects *in vivo* [19].

Furthermore, nanoparticles surface customization can enable target tissues or specific cell surface antigens, targeting specific ligands such as antibody/antibody fragment, peptide, aptamer or small molecules [30].

In the following sections, several important polymers used in the encapsulation of proteins and peptides are summarized.

14.4 Poly Lactic Acid (PLA)

14.4.1 Polymer Structure and Main Characteristics

Polylactic acid (PLA) is an aliphatic ester of lactic acid derived from renewable resources such as corn starch or sugarcane, whose structure is shown in Figure 14.5. It is a biodegradable polymer which has gained commercial interest due to its easy manufacturing [84].

Because of the presence of two chiral centers, lactic acid is a chiral molecule, and therefore it has two stereoisomers: L- and D-lactic acid. These two optical isomers can be produced in different ways; they can be synthesized biologically or chemically [31].

In the biological synthesis, lactic acid is obtained by fermentation of carbohydrates by lactic bacteria belonging mainly to the genus *Lactobacillus,* or fungi [32,33]. This kind of synthesis leads mainly to poly(L-lactic acid). On the other hand, the chemical synthesis could lead to various ratios of L- and D-lactic acid.

The properties of PLA depend on their structure and molecular characteristics. The main factors affecting its behavior are; crystallinity, morphology, crystalline thickness, degree of chain orientation and spherulite size [34].

Because D and L monomers are optically active, the polymers derived from them are semicrystalline. Meanwhile, the optically inactive monomer D,L-PLA is amorphous, which enhanced a homogenuous dispersion of the biological active substance within a monophasic matrix, and for this reason it is applied in drug delivery developments [35].

Polylactic acid shows great potential among biopolyesters, particularly for packaging [36–38] and medical applications, and the reason for this fact is its large availability on the market and its relatively low price [34,36–39].

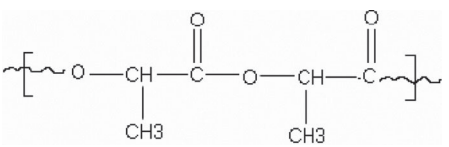

Figure14.5 Chemical structure of Polylactic acid.

Because of its attractive mechanical properties, renewability, biodegradability and relatively low cost, PLA has been the frontrunner in the new generation biopolymers [40].

14.4.2 Encapsulation of Peptides/Proteins in PLA

14.4.2.1 Encapsulation Methods Used

Usually, the encapsulation methods for proteins in PLA are modifications of two basic techniques: solvent extraction/solvent evaporation and spray drying [41,42].

For hydrophilic proteins and peptides the double emulsion/solvent evaporation method is considered as the most convenient because of its encapsulation efficiency and biologically active stability [43]. However, the double emulsion/solvent evaporation method posesses several liabilities such as the required use of toxic solvents that are not easy to fully eliminate (hydrophobic and halogenated solvent) and the inadequate protein release profile. [44–46]. Furthermore, the production of acid degradation products, particularly lactic acid, is the primary instability source for the encapsulated acid-labile protein or peptide [6,8].

One important point is to add additives to prevent proteins from denaturation. The main reason for denaturation of proteins during the preparation of PLA microcapsules by water/oil/water (W/O/W) emulsion is the contact of the protein with the oil/water interface. However, adding protective and stabilizing agents to the inner aqueous phase of W/O/W emulsion can avoid protein drugs from aggregation and denaturation.

Although there are several possibilities, the mechanism of the additives to protect proteins is yet unsolved. One option is that the protection is a result of the competition of the additive with the biological active for absorption on W/O interface, thereby preventing the protein from aggregation and denaturation at the interface. Another option could be that the additive covers the hydrophobic sites of the protein to debilitate the interaction between the protein and the interface [47].

Another clever strategy is to shorten the contact time of the drug with the oil/water interface in order to decrease denaturation and thus the loss of protein activity. This can be achieved by using ethyl acetate instead of dichloromethane as solvent of PLA to prepare double emulsion. By employing ethyl acetate followed by an addition of a large amount of water into the double emulsion, a rapid diffusion of the solvent can be promoted. Using this technique the contact time of the protein with the oil/water interface will be considerably shortened and a rapid solidification of the particles will be accomplished. This method was used to encapsulate bovine hemoglobin by using ethyl acetate as a solvent [14,48].

Dispersing peptide powder in oil phase to prepare solid/water/oil instead of water/oil/water emulsion would be a effective option to protect the biological active since the protein molecules inside the powder will not be available to be in contact with the interface [14].

Another robust technique for one-step encapsulation of fragile hydrophilic agents like peptides and proteins in PLA is coaxial electrospinning. This method is an innovative extension of electrospinning, which uses two concentrically aligned capillaries to enforce the formation of fibers or particles with a core-shell structure [49]. Basically this process removes the damaging effects resulting from the direct contact of the proteins

and peptides with organic solvents and severe emulsification conditions. There are other reviews that detail the principles of the technique and its biomedical applications [49,50].

14.4.2.2 Applications of Peptide/Protein Encapsulated in PLA

Microspheres based on poly(d,l-lactic acid) (PLA) and its copolymer with glycolic acid (poly-d,l-lactic-co-glycolic acid or PLGA) as protein delivery systems have been deeply studied [6,50,52].

Polylactic acid as a carrier for bone morphogenetic protein [53] was studied by Saitoh *et al.* by mixing PLA particles with demineralized bone and implanted subcutaneously on the intercostal muscles of rats. After 24 weeks extensive bone and marrow formation were observed, as well as complete absorption and disappearance of the polylactic acid particles [54].

Konstantina N. Nikou investigated the possibility of inducing a Th1 type of immune response *in vivo* by mixing a HER-2/neu synthetic CTL (cytotoxic T lymphocyte) peptide [HER-2/neu (789–797)] with PLA microspheres. Several formulations of the peptide were administered to HLA-A2.1 transgenic mice. A potent immune response was induced when the *in-vivo* administration of peptide antigen-PLA microparticles was performed. The response observed was comparable to that induced by the combination of the antigen in complete Freund's adjuvant [55].

Poly(ethylene glycol) (PEG)-coated poly(lactic acid) (PLA) nanoparticles were synthesized and tested for their ability to load tetanous toxoid, to deliver it in an active form, and to transport it across the nasal and intestinal mucosa. It was observed that PEG coating improved the stability of PLA nanoparticles in the gastrointestinal fluids and helped the transport of the encapsulated tetanus toxoid across the intestinal and nasal mucosa. Aditionally, a high and long-lasting immune response was reported after intranasal administration of this nanoparticulate formulation [56].

Guanghui Ma clearly shows how hydrophilic poly(lactide)–poly(ethylene glycol) (PELA) microparticles with uniform size and containing recombinant human growth hormone (rhGH) can increase animal weight more apparently only by a single dose and maintain higher blood drug concentration for 2 months compared with PLA and PLGA carriers [14].

A study performed by Saurabh Dixit *et al.* paves the way for the development of a vaccination strategy against *Chlamydia trachomatis,* the most frequently reported sexually transmitted bacterial infection worldwide. It spotlights the potential of a PLA-PEG-based nanoparticle formulation containing a major outer membrane protein of *Chlamydia trachomatis* in inducing a sustained enhanced immune response [57].

14.4.2.3 Biocompatibility

The assessment of the biocompatibility of biodegradable polymers takes into account the incidence of the inflammatory and healing responses of the injected and implanted biomaterials [58].

Studies to date indicate that PLA delivery devices containing bioactive agents are biocompatible and when used in therapeutic applications *in vivo* do not exhibit untoward reactions either locally or systemically [54].

PLA and its copolymers can be used safely for oral, nasal, pulmonary, parenteral, transdermal and intraocular routes of administration [27].

14.4.2.4 Biodegradability and Release

A hydrolytic mechanism governs the degradation process of aliphatic polyester, such as PLA, delivery devices [59–61].

There are several factors that affect the hydrolytic degradation of PLA such as water permeability and solubility (hydrophilicity/hydrophobicity), chemical composition, mechanism of hydrolysis (noncatalytic, autocatalytic, enzymatic), additives (acidic, basic, monomers, solvents, drugs), morphology (crystalline, amorphous), device dimensions (size, shape, surface-to-volume ratio), porosity, glass transition temperature (glassy, rubbery), molecular weight and molecular weight distribution, physicochemical factors (ion exchange, ionic strength, pH), sterilization and site of action [54].

The degradation rate will depend in a considerable way on the crystallinity of the polymer. PLA is highly crystalline and for this reason erodes slower than other similar polymers of its family like PGA or PLGA [58].

In-vivo degradation of PLA was detailed by Pitt *et al.,* indicating that the first stage in the biodegradation process was bound to a decrease in the molecular weight produced by random hydrolytic ester cleavage. Meanwhile, the second stage involved the onset of weight loss and a change in the rate of chain scission [62].

Copolymers of PLA can show different degradation rates depending on their composition; 50:50 lactide/glycolide copolymers have a degradation half-life around 50 to 60 days, meanwhile, 65:35, 75:25 and 85:15 lactide/glycolide copolymers have progressively longer degradation half-lives *in vivo* [58]. Another extremely valuable and highly used PLA copolymer involves poly(lactide)–poly(ethylene glycol) combination. PLA-PEG copolymers posses several advantages, especially when this kind of polymer is used as a matrix of particulate drug carriers, but specifically regarding the biodegradation and the hydrophilic PEG sequence enhanced water uptake, leading to a faster degradation of the kinetics of PLA block [14].

14.4.2.5 Regulatory Status

The hydrolysis of PLA leads to metabolite monomer lactic acid that is endogenous and easily metabolized by the body via the Krebs cycle [27,63]. PLA and its copolymers that contain glycolide (PLGA) have been approved by the FDA for the purpose of drug delivery [27,64].

Polylactic acid's nontoxicity and non-immunogenicity makes it a popular biocompatible polymer that has the approval of the Food and Drug Administration (FDA) for its use as excipient in injections in humans [6,14].

14.5 Poly(lactic-co-glycolic acid) (PLGA)

14.5.1 Polymer Structure and Main Characteristics

Poly(lactic-co-glycolic acid) is a copolymer which is used in a host of Food and Drug Administration (FDA) approved therapeutic devices, owing to its biodegradability and

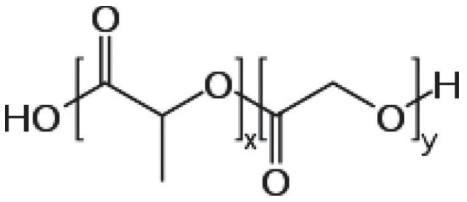

Figure 14.6 Chemical structure of poly(lactic-co-glycolic acid).

biocompatibility. PLGA is synthesized by means of ring-opening copolymerization of two different monomers, the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid. Its chemical structure is shown in Figure 14.6. Polymers can be synthesized as either random or block copolymers, thereby imparting additional polymer properties. Common catalysts used in the preparation of this polymer include tin(II) 2-ethylhexanoate, tin(II) alkoxides, or aluminum isopropoxide. During polymerization, successive monomeric units (of glycolic or lactic acid) are linked together in PLGA by ester linkages, thus yielding linear, aliphatic polyester as a product.

Amongst all the biomaterials, application of the biodegradable polymer poly(lactic-co-glycolic acid) (PLGA) has shown immense potential as a drug delivery carrier and as scaffolds for tissue engineering [65–67]. PLGA is one of the most successfully used biodegradable polymers because its hydrolysis leads to metabolite monomers, lactic acid and glycolic acid. Because these two monomers are endogenous and easily metabolized by the body via the Krebs cycle, a minimal systemic toxicity is associated with the use of PLGA for drug delivery or biomaterial applications [15]. PLGA is approved by the US FDA and European Medicine Agency (EMA) in various drug delivery systems in humans. The polymers are commercially available with different molecular weights and copolymer compositions. The degradation time can vary from several months to several years, depending on the molecular weight and copolymer ratio [68]. The forms of PLGA are generally identified by the monomers ratio used; for example, PLGA 50:50 refers to a copolymer whose composition is 50% lactic acid and 50% glycolic acid.

14.5.2 Encapsulation of Peptides/Proteins in PLGA

14.5.2.1 Encapsulation Methods Used

There exist different methods to prepare nanoparticles that produce several structural organizations. The drug could be entrapped inside the core of a "nanocapsule" or entrapped in or adsorbed on the surface of a matrix "nanosphere" (Figure 14.7).

The most common technique used for the preparation of PLGA nanoparticles is the emulsification-solvent evaporation technique. This technique allows the encapsulation of hydrophobic drugs and consists of dissolving the polymer and the compound in an organic solvent. The emulsion oil (O) in water (W) is prepared by adding water to a polymer solution. The nanosized droplets are induced by sonication or homogenization. The solvent is then evaporated or extracted and the nanoparticles collected after centrifugation [69,70].

A modification of this technique, the double emulsion W/O/W, was used to encapsulate hydrophilic drugs, such as peptides, proteins and nucleic acids. Nanoparticles can

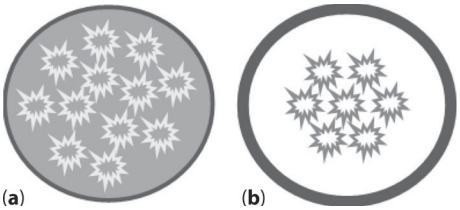

Figure 14.7 Scheme of different types of non-targeted nanoparticles: a) nanospheres and b) nanocapsules.

also be formed by the nanoprecipitation method, also called the interfacial deposition method [71]. There are other available techniques such as the spray-drying method [69]. Drug loading into nanoparticles is achieved by two methods: (i) the incorporation of the drug during the nanoparticles production and (ii) the adsorption of the drug on nanoparticles after their production.

14.5.2.2 Applications of Peptid /Protein Encapsulated in PLGA

The encapsulation of therapeutic proteins in PLGA nanoparticles is an interesting alternative way to overcome the problems related with the administration of proteins alone and also gives other benefits. The incorporation of the proteins into PLGA matrix make available protection against its enzymatic and hydrolytic degradation *in vivo*, maintain their integrity and activity, could improve their bioavailability and in some cases could target the therapeutic protein to the target area [72].

Improved delivery of synthetic peptides and biotechnology-derived medicines is critical to understand the potential of this kind of drug class. It is important to note that sorption of peptides is dependent on polymer-specific molecular properties. Injectable formulations, which deliver peptides from biodegradable PLGA, reduce injection frequency anywhere from once weekly to twice-a-year for treatment of different pathologies such as cancer, endometriosis and diabetes [51].

Listed below are several proteins that have been successfully encapsulated in PLGA nanoparticles.

a. **Endostar** (anti-angiogenic peptide) is an endostatin which shows activity against solid tumors *but* needs multiple injections at a high dose to achieve a therapeutic effect. When PEG-PLGA nanoparticles were loaded with endostar, their half-life became higher than free drug and displayed accelerated inhibition rate and stronger anti-angiogenic effect.
b. **Recombinant human granulocyte colony-stimulating factor (rh-GCSF)** was successfully loaded in PLGA nanoparticles. The obtained formulation showed an *in-vitro* sustained release with 90% of encapsulated protein released from the nanoparticles during the first week [73].
c. **A new GnRH antagonist (degarelix)** was encapsulated in biodegradable PLGA microparticles [74]. Synthetic analogues of gonadotropin-releasing hormone (GnRH) are recognized as potent drugs for sex hormone-dependent diseases, most commonly for prostate cancer, but they are

also indicated for endometriosis, uterine fibroids, *in-vitro* fertilization, and precocious puberty. Degarelix-loaded PLGA microparticles with various peptide loadings were prepared by both solvent (double emulsion solvent evaporation and spray-drying) and non-solvent methods (ram- and micro-extrusion) and the characteristics of loaded particles obtained from each method were determined and analyzed.

d. **PE38KDL, a model protein toxin**, was also loaded in PLGA nanoparticles. *In-vitro* and *in-vivo* data showed that PE38KL-loaded nanoparticles exhibited better *in-vitro* cytotoxicity. In addition, *in vivo*, a better antitumor activity and a higher maximal tolerated dose were observed, showing a decrease of non-specific toxicity due to nanoencapsulation [75].

There are many more examples in the literature of successful encapsulation of protein and peptides in PLGA micro- and nanoparticles.

14.5.2.3 Biocompatibility

As was previously explained, the assessment of the biocompatibility of biodegradable polymers takes into account the incidence of the inflammatory and healing responses of the injected and implanted biomaterials [58]. Research has pointed out that PLGA delivery devices that contain different agents are biocompatible and do not exhibit undesirable reactions when used in therapeutic applications *in vivo.*

14.5.2.4 Biodegradability and Release

A fundamental understanding of the *in-vivo* biodegradation phenomenon of biodegradable polymeric microspheres is one of the important components in the design and development of biodegradable microspheres containing bioactive agents for therapeutic application [3].

PLGA-nanoparticles are internalized in cells in two different ways:

a. Partly through fluid phase pinocytosis; and also
b. Through clathrin-mediated endocytosis.

PLGA-nanoparticles rapidly escape the endo-lysosomes and enter the cytoplasm within 10 minutes of incubation, which facilitates interactions of nanoparticles with the vesicular membranes, leading to transient and localized destabilization of the membrane resulting in the escape of nanoparticles into the cytosol [76].

It is also important to mention that it is possible to tune the physical properties of the polymer-drug matrix by controlling the relevant parameters: polymer molecular weight, ratio of lactide to glycolide and drug concentration to achieve a desired dosage and release interval depending upon the drug type [77–79].

The PLGA (drug delivery specific vehicle) must be able to deliver its load with appropriate extent of time, concentration and biodistribution for the planned therapeutic effect. So some relevant parameters of the design are the material, the geometry and location that must incorporate mechanisms of degradation and approval of the

vehicle, as well as active pharmaceutical ingredients. It is known that the biodistribution and the pharmacokinetics of PLGA display a nonlinear and dose-dependent behavior [80]. Furthermore, previous studies suggest that both blood clearance and uptake by the mononuclear phagocyte system (MPS) may depend on the dose and composition of PLGA carrier systems [81]. Additionally, whole-body autoradiography and quantitative distribution experiments indicate that some formulations of PLGA, such as nanoparticles, accumulate rapidly in different parts of the human system such as: bone marrow, spleen, lymph nodes, liver, and peritoneal macrophages. The degradation of the PLGA vehicles inside the human body is quick on the first or initial step (near to 30%) and slows eventually to be cleared by respiration in the lung [82]. To address these limitations, studies have investigated the role of surface modification, suggesting that incorporation of surface modifying agents can significantly increase blood circulation half-life [83]. PLGA suffers degradation by hydrolysis or biodegradation through cleavage of its backbone ester linkages into oligomers, and finally, monomers which has been proved in *in-vivo* and *in-vitro* tests and for different drugs and proteins [84].

The change in PLGA properties during polymer biodegradation influences the release and degradation rates of incorporated drug molecules. The degradation process of PLGA is mainly through uniform bulk degradation of the matrix; the water uptake is higher than the rate of polymer degradation. Besides, the increase of carboxylic end groups as a result of biodegradation autocatalysis in the process. The degradation of PLGA copolymer is the collective process of bulk diffusion, surface diffusion, bulk erosion and surface erosion. The biodegradation rate of the PLGA copolymers depends on:

a. ***The molar ratio of the lactic and glycolic acids in the polymer chain***: Polymer composition is the most important factor that determines the hydrophilicity and rate of degradation of the matrix. The increase in glycolic acid percentage produces an increase in the rate of weight loss of polymer;
b. ***The molecular weight of the polymer***: Polymers with higher molecular weight exhibited lower degradation rates because the molecular weight has a direct relation with the polymer chain size [85];
c. ***The degree of crystallinity***: It has been proposed that this parameter produces a decrease of degradation rate; and
d. ***The glass transition temperature (Tg) of the polymer***.

The drug release from PLGA-based matrix is a combination of surface diffusion, bulk diffusion, and erosion of the matrix, which can be associated to several physical, chemical and processing parameters of the particular system. Nonetheless, fundamental mechanisms of this intricate process have not been clearly understood until now. The first stage of drug release takes place through random scission of the polymer; at this stage no polymer weight loss occurs and the main process is the diffusion. In contrast, the second stage is related to the beginning of weight loss. One of the most famous diffusion models of polymer degradation incorporates the effect of polymer degradation on the drug diffusivity in the polymer and the non-uniform distribution of that drug inside the formulation [86].

14.5.2.5 Regulatory Status

Poly(lactic-co-glycolic acid) (PLGA) has been approved by the US Food and Drug Administration (FDA) and European Medicine Agency (EMA) in various drug delivery systems in humans. The polymers are commercially available with different molecular weights and copolymer compositions. The degradation time can vary from several months to several years, depending on the molecular weight and copolymer ratio [68].

Although PLGA has been shown to be extremely safe as a material for macroscopic and microparticle systems, unique considerations may arise when using nanoscale applications. Several studies suggest that nanoparticles of any material may create specific biodistribution and toxicological profiles [87].

14.5.2.6 Advantages and Disadvantages of PLGA

Advantages

The success of any medical treatment depends on two important factors:

- The pharmaco-kinetic/pharmaco-dynamic activity of the therapeutic agent,
- The bioavailability at the site of action in the human system.

In this context, PLGA is a widely used polymer for fabricating NPs because of biocompatibility, a long-standing track record in biomedical applications and well-documented utility for sustained drug release compared to the conventional devices of up to days, weeks or months, and ease of parenteral administration via injection.

PLGA-based nanoparticles present many advantages for drug delivery. They can protect drugs from degradation and enhance their stability. Moreover, due to their size, nanoparticles can penetrate specific tissues via:

- The fenestrations present in the endothelium of cancer and inflamed tissue; or
- The receptors overexpressed by target cells or in the blood brain barrier. This allows a specific delivery of drugs, proteins, peptides or nucleic acids to their target tissue. PLGA-based nanoparticles can increase the efficacy of treatments because of the sustained release of the therapeutic agent from stable nanoparticles. They can improve pharmacokinetic and pharmacodynamic profiles.

Another major advantage of PLGA over other polymers is that PLGA is approved by the FDA and EMA in various drug delivery systems, putting PLGA-based nanoparticles in a good position for clinical trials.

Disadvantages

PLGA-based systems also present some disadvantages:

a. The low drug loading described for many drugs;
b. The high cost of production; and
c. The difficulty of the scale-up.

In order to commercialize a new drug delivery system, the economic aspect has to also be analyzed for the pharmaceutical industry but also for patients. The production of GMP grade of PLGA with specific selected properties can be very expensive. In addition, another limitation for the commercialization of nanoparticles is the scaling-up. There are many steps involved in the laboratory-scale production that are impossible to reproduce industrially.

14.6 Chitosan

14.6.1 Chitosan Structure and Main Characteristics

Chitosan, an acid soluble and renewable biopolymer, was first studied as a drug delivery agent in 1990 [88]. It is a natural polymer that has been used extensively in the medical field (tissue engineering, wound healing, drug delivery, among others) [89–91]. Chitosan is the most important derivative of chitin, obtained by (partial) deacetylation of chitin in the solid state under alkaline conditions or by enzymatic hydrolysis in the presence of chitin deacetylase (Figure 14.8). As chitin occurs naturally (for example in fungal cell walls and crustacean shells), chitosan is a fully biodegradable, biocompatible and nontoxic natural polymer [92].

Chitosan is soluble in aqueous acidic media. Solubilization takes place when the $-NH_2$ function on the C-2 position of the D-glucosamine repeat unit is protonated. Chitosan is the only pseudonatural cationic polymer, and thus it finds many applications that follow from its unique character (flocculants for protein recovery, depollution, etc.). Being soluble in aqueous solutions, it is largely used in different applications as solutions, gels, or films and fibers. In the solid state, chitosan is a semicrystalline polymer. The degree of crystallinity and the size of the crystals depend on the experimental conditions (molecular weight, degree of acetylation, methods of preparation) [93].

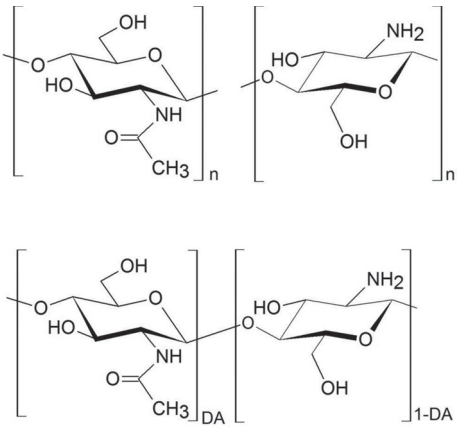

Figure 14.8 Chemical structure of different chitosans.

14.6.2 Encapsulation of Peptides/Proteins

14.6.2.1 Encapsulation Methods Used

Chitosan-based particles loaded with proteins can be obtained by both physical and chemical methods. However, the chemical crosslinking methods use toxic organic solvents (to make water/oil emulsions), heat, vigorous agitation or chemicals that might compromise the stability and biocompatibility of the proteins [93].

14.6.2.2 Chemical Crosslinking Methods

Glutaraldehyde is the most common crosslinker used. The mechanism to produce crosslinking involves the formation of Schiff's base via nucleophilic attack by the nitrogen of the amino group (from chitosan) on the carbon of the glutaraldehyde, which displaces the oxygen of the aldehyde, resulting in the C-N bond [94]. However, it is well known that glutaraldehyde (like many chemical crosslinkers) can impart toxicity, which may result in the decline of biocompatibility [95]. Other chemical crosslinkers are: Genipin (an excellent crosslinker for polymers containing amino groups) [96], Glyoxal (can crosslink chitosan in the same way as glutaraldehyde), Dextran Sulfate and Bifunctional Crosslinking Agents such as epichlorohydrin, diisocyanate, or epoxy compounds, or ethylene glycol diglycidyl ether, among others [97].

14.6.2.3 Physical Crosslinking Methods

A technique used to obtain spherical particles is the spray-drying technique. The spray-drying method has reportedly provided small-size, protein-loaded chitosan-alginate microparticles with appropriate protein immobilization capacity [98]. It has been applied to BSA/sodium alginate/chitosan solutions to obtain microparticles [98]. Moreover, chitosan nanoparticles can be obtained by ionic crosslinking method. It has produced nanoparticles based on ionic gelation process of tripolyphosphate (TPP) and chitosan [99]. Nevertheless, another physical method called layer-by-layer can be used to form chitosan particles. Through the layer-by-layer technique submicron (40 nm) capsules with poly(L-aspartic acid) and chitosan for transmucosal delivery of proteins and peptides have been obtained [100].

14.6.2.4 Applications of Peptide/Protein Encapsulated in the Polymer

Peptides and proteins are generally administered by the parenteral route. However, complications such as thrombophlebitis or tissue necrosis and poor patient compliance have stimulated the investigation of alternative nonparenteral routes [101].

The oral administration of peptidic drugs requires their protection from degradation in the gastric environment and the improvement of their absorption in the intestinal tract. In general, the barriers can be divided into the absorption and the enzymatic barrier, which are mainly responsible for a very low bioavailability of orally given peptides and proteins. Because of i) their permeation enhancing effect, ii) enzyme inhibitory capabilities, and iii) mucoadhesive properties, chitosan and

its derivatives are able to reduce both barriers, which makes these polymers important excipients for peroral peptide delivery systems [102]. Chitosan-based delivery systems enhance the absorption and/or cellular uptake of peptides/proteins across mucosal sites and have immunoadjuvant properties. Chitosan is a mucoadhesive polysaccharide capable of opening the tight junctions between epithelial cells and it has functional groups for chemical modifications, which has resulted in a large variety of chitosan derivatives with tunable properties for the targeted applications [103].

14.6.2.5 Biodegradability and Release

Currently, there are still limited, incomplete, and insignificant human studies about the biocompatibility and biodegradability of newly developed chitosan derivatives. There are limited studies published in peer-reviewed journals describing the biocompatibility of chitosan or its derivatives, due to an absence of interest from manufacturers in applications of chitosan or its derivatives in the design of medical devices [104].

Chitosan can be degraded by enzymes able to hydrolyse glucosamine–glucosamine, glucosamine–N-acetyl-glucosamine and N-acetyl-glucosamine–Nacetyl-glucosamine linkages [105]. Chitosan is thought to be degraded in vertebrates predominantly by lysozyme and by bacterial enzymes in the colon [106]. It has been found that both the rate and extent of chitosan biodegradability in living organisms are dependent on the degree of deacetylation [107]. In *in-vitro* assays, lysozyme has been found to efficiently degrade chitosan [106]

Other modifications (such as covalent crosslinking and thiolation) have been shown to alter degradation profiles [108]. Also, a variety of proteases were found to degrade chitosan films [109].

14.6.3 Peptides and Proteins Encapsulated in Chitosan

14.6.3.1 Bovine Serum Albumin (BSA)

Bovine serum albumin is the most common protein used as a model protein. In general, researchers first carry out the *in-vitro* assays with this protein [98]. It has been reported that microencapsulated beads composed of alginate blended with a water-soluble chitosan have been evaluated for delivery of BSA [110].

14.6.3.2 Insulin

The possibility of administering insulin via oral or nasal routes has been investigated over the years, but the major complications for insulin are the enzymatic degradation and poor transmucosal absorption. The biopolymer chitosan and its derivatives or salts have been widely studied in oral insulin delivery and nasal or transdermal routes. Recent patented strategies on insulin delivery are based on chitosan for its mucoadhesive, which is able to protect the insulin from enzymatic degradation, prolong the retention time of insulin, as well as open the interepithelial tight junction to facilitate systemic insulin transport [111]. Absorption of insulin is physically hindered by the absorption barrier consisting of a single layer of columnar epithelial cells joined at the apical surface by a tight junction complex. The presence of negative charges in the

junction complex leads to segregation of the apical layer from the basolateral compartment of the epithelial cells, making the intestinal environment selective for particles based on size and charge. Chitosan nanoparticles are capable of delivering insulin, overcoming these barriers [112]. This polymer is capable of being chemically changed to produce water-soluble, low molecular weight chitosan, which renders insulin able to be processed under mild conditions; or it can be obtained as a sulphated chitosan, which opens the paracellular channels for insulin transport. Also, a combination of chitosan and fatty acid as hydrophobic nanoparticles can promote the insulin absorption via lymphoid tissue [111]. Moreover, it was reported that acidic solutions of chitosan salts have been shown to increase the nasal absorption of insulin *in vivo* [113]. Also, chitosan nanoparticles have been coated with PLGA and it has been reported that the stability of the particles in the presence of lysozyme was improved and the nasal transport of the encapsulated tetanus toxoid was enhanced. Moreover, these nanoparticles were very efficient in improving the nasal absorption of insulin as well as the local and systemic immune responses to tetanus toxoid following intranasal administration. In conclusion, these nanoparticles increase the prospects of their usefulness for mucosal protein delivery and transport [56]. It has been found that insulin-loaded chitosan nanoparticles administered orally to diabetic rats reduced their glucose levels to a normal range for more than several hours [114,115].

14.6.3.3 Peptides in the Central Nervous System

The problems associated with the use of peptides in the central nervous system are that they have short duration of action, have to overcome the blood brain barrier and need parenteral administration. However, in the last few years there have been significant advances in delivering peptides. Noninvasive methods of delivering peptides to the brain include the use of chitosan amphiphile nanoparticles for oral and nasal delivery routes. Examples of this are chitosan amphiphile nanoparticles developed by Nanomerics Ltd., cell penetrating peptides, and the utilization of nose to brain strategies by a number of companies which are also recording notable successes [116]. Moreover, delivery of therapeutic proteins to brain tissue is further limited by the size and physicchemical properties of proteins. A chitosan-conjugated Pluronic-based nanocarrier has been developed with a specific target peptide for the brain (rabies virus glycoprotein; RVG29) and applied for the protein delivery to the brain. It has been found that the delivered protein in the brain maintained its bioactivity. Therefore, RVG29- and chitosan-conjugated Pluronic-based nanocarrier could be potentially useful for the diagnosis and therapy of brain diseases [117].

14.6.3.4 Salmon Calcitonin

Inhalable co-spray dried powders of salmon calcitonin-loaded chitosan nanoparticles with mannitol have been prepared and analyzed. Salmon calcitonin or Calcitonin (also known as thyrocalcitonin) is a 32-amino acid linear polypeptide hormone that is produced in humans primarily by the parafollicular cells (also known as C-cells) of the thyroid, and in many other animals in the ultimobranchial body. It acts to reduce blood calcium (Ca^{2+}), opposing the effects of parathyroid hormone (PTH) [118]. The

nanoparticles have been obtained by the ionic gelation method using sodium tripolyphosphate as a crosslinking polyion. It was reported that the chitosan-drug system has appropriate aerodynamic properties for pulmonary delivery and was able to deliver salmon calcitonin via a pulmonary route into the systemic circulation [119].

14.6.3.5 Regulatory Aspect

The FDA approved chitosan only in the management of minor topical bleeding wounds such as minor abrasions and minor skin lacerations (http://www.fda.gov/default.htm). Chitosan is not approved by the FDA for any product in drug delivery, and as a consequence very few biotech companies are using this material [120].

14.7 Final Comments and Future Perspectives

Due to their biocompatibility and biodegradability, PLA, PLGA and chitosan have been shown to be excellent delivery carriers for controlled administration of drugs, peptides and proteins.

Improved delivery of synthetic peptides and biotechnology-derived medicines are critical to understand the potential of this kind of drug class. It is important to note that sorption of peptides is dependent on polymer-specific molecular properties. In general, the degradation and the drug release rate depend on the hydrophilicity, the chemical interactions among the hydrolytic groups, the crystallinity and the volume-to-surface ratio of the device. All of the factors must be considered in order to tune the degradation and drug release for the selected application. Furthermore, multiple studies demonstrate that all of the studied polymers can easily be formulated into the drug carrying devices at all scales, micro and nano, from which peptides or proteins can be delivered over different periods of time with diverse routes of delivery.

Another important point to be taken into account is the toxicology of the generated carriers, especially in the nanometer-size scale. A new subdiscipline known as "nanotoxicology" has come into sight because the interactions of nanocarriers with biological systems are really complex and the size and surface properties of nanocarriers modify the behavior of these components in the body. Toxicology studies and regulations are necessary in order to fully define the biocompatibility of nanocarriers in humans.

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15

Eco-Friendly Grafted Polysaccharides for Pharmaceutical Formulation: Structure and Chemistry

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Abstract

Drug formulations have played an important role in pharmaceutical industries over many years. These formulations are used in the different fields of pharmacy like controlled drug delivery, ocular drug delivery, pulmonary drug delivery etc. Various types of methods are available for the formulations of drugs. Generally, nontoxic and biodegradable polymers are used for these formulations. The natural polysaccharides are gaining major importance in pharmacy because of their low cost, easy availability and no risk to health. The modified polysaccharides by simple graft copolymerization process are much more effective than the original polysaccharides. Grafted polysaccharides are generally used for drug formulations. In this review, we discuss the recent trends of controlled drug delivery by polymeric matrix systems and microsphere systems using grafted natural polysaccharides.

Keywords: Drug formulations, controlled drug delivery, natural polysaccharides, graft copolymers

15.1 Introduction

In recent years, controlled drug delivery formulations and the polymers used in these systems have become much more sophisticated, with the ability to achieve the effective release period for a particular drug. The field of natural polymer modification is a rapidly developing area because of its wide range of applicability in drug delivery, biomaterials development, food and beverages, water purification, paper, textiles, fuel cells, etc. Natural polysaccharides are unique raw materials because of their wide availability, renewability, stability, hydrophobicity and modifiable nature. They offer tremendous potential for the development of alternate drug delivery, biomaterial and water purification systems [1–5]. Over the past few decades, the rise of modern pharmaceutical technology and the amazing growth of the biotechnology industry have revolutionized the approach to drug delivery systems development [6]. Pharmaceuticals have

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primarily consisted of relatively simple, fast-acting chemical compounds that are dispensed orally (as solid pills and liquids) or injected. A variety of controlled release (CR) systems have been investigated in order to decrease dosing frequency, enhance patient compliance, reduce the plasma concentration fluctuation in drug levels and facilitate more uniform release of drug over an extended period of time. Such formulations are quite convenient to deliver the drug in a more controlled and predetermined manner than the conventional type of dosage formulations which are needed to maintain the therapeutically required plasma concentration of the drug during its systemic circulation for a prolonged time [7,8]. Such advantages are successfully met by using the biocompatible and biodegradable polymers, in which the active pharmaceutical ingredient can be either physically dispersed or covalently bonded to the polymer bac kbone, such that the release of drug occurs by diffusion mechanism through the polymer wall [9]. Among all the methods of develop g CR systems, microspheres have emerged as the most attractive formulation strategies [10] as these provide many advantages over the CR tablets in view of their distribution following predictable and controllable release kinetics [11]. Drug formulation research fields have focused on the systems that delay the release of drugs from substrate for years. The interest in the drug formulation release systems arises from the requirements for achieving slow release of highly water-soluble compounds, directing such compounds to the target organ or cell, achieving release rates that match given objectives, decreasing the number of daily applications, improving compliance and minimizing side effects [12]. Recently, the design and synthesis of graft copolymers and functional hollow microspheres with nano/micrometer dimensions, unique properties and an environmental response have attracted a great deal of attention owing to their potential applications in encapsulation and controlled release (CR) systems for drugs, gene delivery and protective shells for cells and enzymes [13–18]. During the past decades research has been going on in developing methods to target the drug to the specific region. The goal of targeted drug delivery is to deliver the drug to the specific organ. Graft copolymers are used as matrix tablets for prolonged-release of drugs via oral mode due to their simple and low-cost manufacturing process. It is important to maintain the therapeutic level of any drug which is administered in the body for treatment. Polymers are becoming increasingly important in pharmaceutical applications especially in the field of drug delivery. Polymers range from their use as binders in tablets to viscosity and flow controlling agents in liquids, suspensions and emulsions. Many people around the world are benefiting from advanced drug delivery systems, receiving safer and more effective doses of medicines.

15.1.1 Targeted Drug Delivery

Targeted drug delivery is a process in which concentration of medicine is increased in the specified part of the body rather than in other parts after its administration. The objective is to protect drug interaction from diseased tissue and supply a definite quantity for an extended period within the body.

15.1.2 Controlled Drug Delivery

Advances in controlled release drug delivery systems have been largely based on advances in functional polymers. Controlled release drug delivery technologies have

made quantum advances in the last three decades from primitive delayed release dosage forms in the 1960s to highly sophisticated self-regulated delivery systems in the 1990s [6]. Drugs can now be delivered at zero-order for periods ranging from days to years. Such technological advances have produced many clinically useful controlled release dosage forms and have provided new life for many existing polymers.

15.1.3 Current Status of Controlled Drug Release Technologies

Nowadays, controlled release technologies are dependent upon smart polymers. This current technology of controlled drug delivery is primarily based on polymers having various properties. The history of controlled drug delivery has been based on the evolution of polymers. Simple pH-sensitive polymers are commonly used for enteric coating of so-called "smart" polymers used in self-regulated (or modulated) drug delivery systems. Polymers have made it possible to design controlled release dosage forms with a variety of release profiles [19]. It is the polymer that has also made it possible to deliver heterologous cells releasing desired bioactive agents without immune responses in humans [20]. Undoubtedly, future advances in controlled drug delivery will continue to depend on the advances in new polymeric materials, such as synthetic polymers with novel functions [21] and protein mimetics [22]. There are a number of polymers that have been used in drug delivery that are known to be safe, which comprise the generally regarded as safe (GRAS) materials. Various dosage forms for pharmaceutical formulations for controlled drug delivery systems are depicted in Figure 15.1.

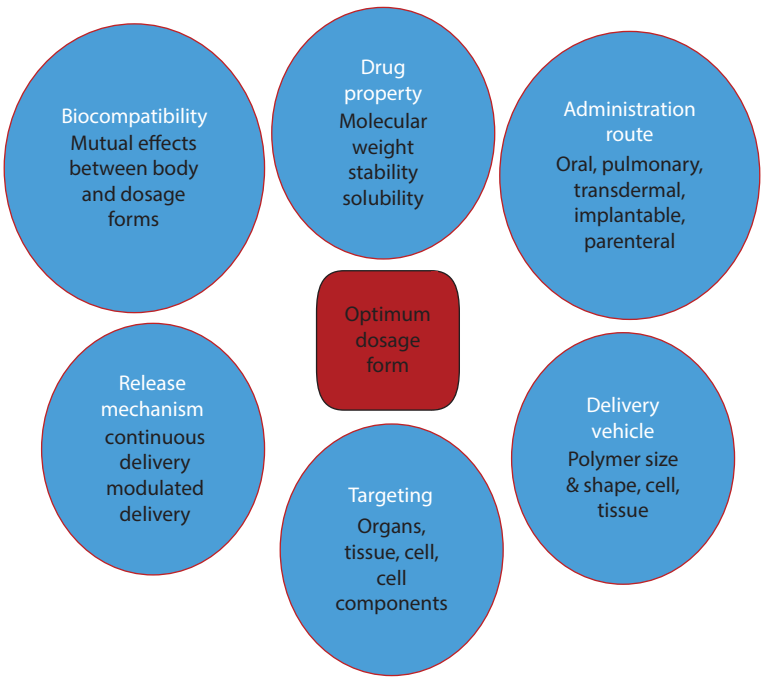

Figure 15.1 Independent factors important in the design of controlled drug delivery systems. The pathophysiology of a disease also plays a critical role in the definition of the final goal of this scheme—development of an optimal dosage form [23].

Controlled drug delivery technology using polymers as carriers including natural or synthetic polymers represents one of the most rapidly advancing areas of science [24–26]. In addition to natural and synthetic polymers, there are many carrier materials for drug delivery which are formed by the combination of natural polymers and synthetic polymers [27–30]. In other words, polymeric materials with various properties can be used for designing a variety of drug delivery systems. Synthetic polymeric microspheres CPVA-g-PSSS for which anionic polyelectrolyte, poly(sodium 4-styrene sulfonate) (PSSS) was grafted onto the surface of cross-linked poly(vinyl alcohol) (CPVA) microspheres to design a novel colon-specific drug delivery system [31]. Over the past few years, oral site-specific drug delivery systems have attracted a great deal of interest, specially colon-specific drug delivery systems have been developed largely for the local treatment of a variety of bowel diseases and for improving systemic absorption of drugs. Various approaches have been reported during the last decade to develop new methodologies for colon-specific drug release, including pH-sensing release systems in which pH-sensitive polymers are used [32–35].

15.1.4 Pharmaceutical Formulation

Pharmaceutical formulation can simply be defined as the design of dosage forms [36]. Currently pharmaceutical formulation must ensure adequate bioavailability, in addition to facilitate drug delivery and stability. In practice, formulating consists of selecting the excipients, the manufacturing process and evaluating the formulations obtained using physicochemical and pharmacotechnical tests. These tests allow selecting, optimizing and evaluating pharmaceutical preparations based on pre-established specifications [37].

15.1.5 Stages and Timeline

Preformulation studies involve characterization of a drug's physical, chemical and mechanical properties in order to choose other suitable ingredients which should be used in the preparation. Formulation studies involve developing a preparation of the drug which is both stable and acceptable to the patient. For orally taken drugs, this usually involves incorporating the drug into a tablet or a capsule. Formulation studies then consider such factors as particle size, polymorphism, pH and solubility, as all of these can influence bioavailability and hence the activity of a drug. The drug must be combined with inactive additives by a method which ensures that the quantity of drug present is consistent in each dosage unit, e.g., each tablet. The dosage should have a uniform appearance, with an acceptable taste, tablet hardness or capsule disintegration. A low drug load may cause homogeneity problems. A high drug load may pose flow problems [38].

15.1.6 Types of Pharmaceutical Formulation

The drug form varies by the route of administration.

15.1.6.1 Enteral Formulation

Oral drugs are normally taken as tablets or capsules. The drug (active substance) itself needs to be soluble in aqueous solution at a controlled rate. Such factors as particle size and crystal form can significantly affect dissolution. Fast dissolution is not always ideal. For example, slow dissolution rates can prolong the duration of action. The most important characteristic for oral formulation is that it must be able to overcome the problems associated with oral administration. The most critical problem is rate of drug solubility, i.e., the active ingredient of the drug must be soluble in aqueous solution at a constant rate [39]. The oral formulation is divided in two parts which are: Tablet form and Capsule form.

15.1.6.1.1 Tablet

A tablet is usually a compressed preparation that contains:

a. 5–10% of the drug (active substance).
b. 80% of fillers, disintegrants, lubricants, glidants and binders.
c. 10% of compounds which ensure easy disintegration, disaggregation and dissolution of the tablet in the stomach or the intestine.

The disintegration time can be modified for a rapid effect or for sustained release. Special coatings can make the tablet resistant to the stomach acids such that it only disintegrates in the duodenum, jejunum and colon as a result of enzyme action or alkaline pH. Pills can be coated with sugar, varnish or wax according to the desired taste [40].

15.1.6.1.2 Capsule

A capsule is a gelatinous envelope enclosing an active substance. Capsules can be designed to remain intact for some hours after ingestion in order to delay absorption. They may also contain a mixture of slow and fast-release particles to produce rapid and sustained absorption in the same dose [40].

15.1.6.1.3 Sustained Release

Some tablets are designed with an osmotically active core, surrounded by an impermeable membrane with a pore in it. This allows the drug to percolate out from the tablet at a constant rate as the tablet moves through the digestive tract [41].

15.1.6.2 Parenteral Formulations

These are also formulations called injectable formulations being used with intravenous, intramuscular and intraarticular administration. The drug is stored in liquid, or if unstable, lyophilized form. Many formulations are unstable at higher temperatures and require storage at refrigerated or sometimes frozen conditions. The logistics process of delivering these drugs to the patient is called the cold chain. The cold chain can interfere with delivery of drugs, especially vaccines, to communities where electricity is unpredictable or nonexistent. These may include lyophilized formulations which are easier to stabilize at room temperature. Most protein formulations are parenteral due to the fragile nature of the molecule which would be destroyed by

enteric administration. Proteins have tertiary and quaternary structures which can be degraded or cause aggregation at room temperature. This can impact the safety and efficacy of the medicine [42].

15.1.6.2.1 Liquid

Liquid drugs are stored in vials, ampoules, cartridges, and prefilled syringes. As with solid formulations, liquid formulations combine the drug product with a variety of compounds to ensure a stable active medication following storage. These include solubilizers, stabilizers, buffers, viscosity enhancers/reducers, surfactants, chelating agents and adjuvant. If concentrated, the drug may be diluted before administration [43].

15.1.6.2.2 Lyophilized

Lyophilized drugs are stored in vials, cartridges, dual chamber syringes, and prefilled mixing systems. Lyophilization or freeze drying is a process that removes water from a liquid drug, creating a solid powder or cake. The lyophilized product is stable for extended periods of time and could allow storage at higher temperatures. In protein formulations, stabilizers are added to replace the water and preserve the structure of the molecule [44]. Before administration, a lyophilized drug is reconstituted as a liquid before being administered. This is done by combining liquid diluents with the freeze-dried powder, mixing and then injecting. Reconstitution usually requires a reconstitution and delivery system to ensure that the drug is correctly mixed and administered [43].

15.1.6.3 Topical Formulations

1. (a) Cream – Emulsion of oil and water in approximately equal proportions, it penetrates startum corneum, outer layer of skin wall; (b) Ointment – Combines oil (80%) and water (20%), an effective barrier against moisture loss; (c) Gel – Liquefies upon contact with the skin; (d) Paste – Combines three agents - oil, water, powder, an ointment in which a powder is suspended; and (e) Powder – A finely subdivided solid substance [44].

15.2 Polysaccharides

Polysaccharides are the naturally occurring polymeric compounds. They are found in all living organisms, various tissues of seeds, leaves of plants, body fluid of animals, insects, cell wall of the crops, etc. The polysaccharides are directly obtained from these sources. Sometimes polysaccharides are extracted from renewable resources like bacteria, yeast and fungi by microbial culture. The seed polysaccharides like psyllium, quince, flax and gum etc., have been utilized for commercial uses in food, textiles, cosmetics and pharmaceutical industries. Polysaccharides are used as a flocculant like starch, guar gum, cassia gum, xanthan gum, barley, sodium alginate, agar and chitosan. The modified forms of these natural polysaccharides are also used as superabsorbent materials for toxic heavy metals, toxic dye removal and controlled release of therapeutic drugs. The modification of these polysaccharides is carried out by the grafting of the

polymeric chains, oxidative or hydrolytic degradation and derivatization of the functional groups [45,46].

15.2.1 Chemistry of Polysaccharides

Polysaccharides are generally macromolecular structures consisting of a large number of monosaccharide units. Sometimes they are also called glycans. Polysaccharides are built up by the combination of sugar units by glycosidic linkages. If the polysaccharides are built up by one kind of sugar unit, they are called homo-polysaccharides (homoglycans), when they are built up by two or more different kinds of sugar units, they are called hetero-polysaccharides (heteroglycans). The homo- and hetero-polysaccharides may be linear or branched structures [47].

Cellulose is an abundant carbohydrate of commercial and biological importance found in all plants as the major structural component of the cell walls. Cellulose in wood is mixed with many other polymers such as hemicelluloses and lignin. It has to be split from these components to be used for paper production. The fluffy fiber found in the cotton ball is the purest naturally occurring form of cellulose. Cellulose is the β–isomer of amylose consisting of β–(1,4)–linked glucose residues. This is shown in Figure 15.2. The different stereochemistry of the glycosidic linkage compared to amylose gives cellulose totally different properties. In contrast to amylose, β–linkages in cellulose allow the polymer to fold in a fully extended conformation to form a sheet-like secondary structure. The tertiary structure of cellulose is characterized by parallel-running intermolecular hydrogen-bonded cellulose chains further associated by hydrogen bonds and van der Waals forces to produce three-dimensional microfibrils. This gives cellulose fibers exceptional strength and makes them water insoluble despite their hydrophilicity. The cellulose microfibrils give an X–ray diffraction pattern that indicates regular, repeating microcrystalline structures interspersed with less-ordered paracrystalline regions. As a consequence of its three-dimensional structure, cellulose cannot be hydrolyzed by starch-degrading enzymes. The cellulose-degrading enzymes, called cellulases, are produced by microorganisms [48].

15.2.2 Grafted Polysaccharides

The modification of polysaccharides is needed to improve the properties of these polysaccharides. Grafting is one of the best methods for the modification of polysaccharides. Graft copolymerization is a process in which side chain grafts are covalently attached

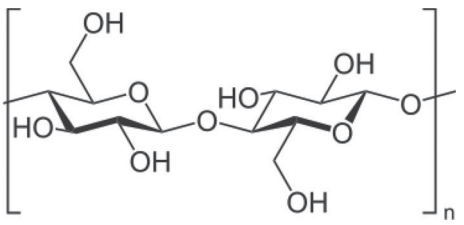

Figure 15.2 Chemical structure of cellulose [48].

to the main chain of polymer backbone to form branched copolymer [49]. The extent of polymerization graft is referred to as the percentage grafting. It can be calculated by the following formula:

$$\%\text{grafting} = \frac{\text{Wt. of graft copolymer} - \text{Wt. of polysaccharide}}{\text{Wt. of polysaccharide}} \times 100 \quad (15.1)$$

Various types of methods have been developed for the graft copolymerization like conventional grafting methods, microwave-assisted grafting methods and microwave-initiated grafting methods. All these techniques have been used to create free radical sites on polysaccharides backbone through the free radicals generation pathway. The rate of grafting reactions depends upon the initiator concentration as well as monomer concentration, time of irradiation and nature of polysaccharides backbone. The free radicals are generated by the use of radical initiators such as redox reagents like Fenton's reagent (Fe^{+2}/H_2O_2), Persulphate, Peroxides, transition metal ions (e.g., Ce^{4+}, Cr^{6+}, V^{5+},Co^{3+}), metal chelates (e.g., $Fe(acac)_3$, $Zn(acac)_2$, $Al(acac)_3$, $VO(acac)_2$), ceric ammonium nitrate, potassium persulphate, ammonium persulphate, etc. Sometimes UV radiations, electron beam, gamma rays and microwave irradiations are used for the generation of free radical sites on the polysaccharide backbone. In the polymerization reaction, the free radicals initiate the reaction process. The free radical polymerization reactions proceed via three steps, i.e., initiation, propagation and termination. In the initiation steps of graft copolymerization reaction the polysaccharides backbone and monomers are initiated by the radicals derived from the initiator. In the propagation step, the addition of monomers to polysaccharides takes place. In the termination process the propagating radicals are combined together to form the graft copolymer. Homopolymers have been formed as side products in the graft copolymerization reaction [50–52].

15.2.3 Drug Delivery System by Grafted Polysaccharides

Graft copolymers are used as matrix tablets for prolonged-release of drugs via oral mode, due to its simple and low-cost manufacturing process. It is important to maintain the therapeutic level of any drug which is administered in the body for treatment. Therapeutic level refers to the concentration of drug in the body tissues, blood, etc., without causing any side effects.

Conventional delivery systems (CDS) are characterized by rapid and uncontrolled drug release kinetics. Here, drug absorption is controlled by the physiological mechanisms which assimilate the therapeutic molecule [53,54].

The side effects due to a drug are directly related to its concentration in the body. The concentration is initially high when the drug is administered in the body, which may lead to unwanted side effects. It is also likely that the subtherapeutic level of drug leads to create gradual resistance of drug in the body. In CDS, the therapeutic level of the drug is maintained by repeated and frequent administration of dosage, which results in discomfort.

Controlled release system (CRS) consists of the drug enclosed in a matrix, which releases it at a predetermined rate. Thus the therapeutic level of drug is also

maintained. The sustained or prolonged release has been long desired and rationalized for decades.

Basically, there are three basic modes of drug delivery:

1. Targeted delivery or spatial distribution of drug: It has the goal of delivering the drug to specific cells, tissues or organs [55].
2. Controlled release or temporal distribution of drug: It releases drug at a predetermined rate, or at specific times or with specific release profiles [55].
3. Modulated release refers to the use of a drug at a variable rate controlled by various factors [55].

Oral mode of drug intake is a natural choice for us. It is not feasible in some cases, like in the case of peptides (e.g., insulin, estrogen and progesterone hormones), where the enzymes in the stomach may degrade it into its constituent amino acids. Also, drugs like acetyl salicylic acid damage the stomach lining and prolonged consumption gives serious side effects. In the human gastrointestinal tract (G.I. tract), pH is acidic in stomach, neutral/alkaline in lower G.I. tract.

Targeted drug release utilizes this variation in pH of our G.I. tract. It can act as a trigger for release of drug from the enclosed matrix. Alternatively, release of a drug in the stomach can be avoided/minimized by enclosing it in a matrix which does not release the drug in acidic pH.

In the field of drug delivery, polysaccharides (such as starch, chitosan, alginate, and cellulose) and modified polysaccharides (e.g., cellulose derivatives) have earned special attention due to their high biocompatibility and hydrophobicity [56–61].

15.2.4 Concept of Drug Delivery Matrix

Controlled release of a drug has been achieved till now by enclosing it in a hydrophobic matrix (wax, polyethylene, polypropylene, and ethyl cellulose) or hydrophilic matrix (such as carboxymethyl tamarind, hydroxyl propyl cellulose, hydroxyl propyl methylcellulose, methylcellulose, sodium carboxymethylcellulose and alginates) [62,63].

Various techniques of drug loading into the matrix are in practice. Some of the most commonly used are:

1. Solvent swelling technique: The matrix can be left to swell in the highly concentrated drug solution. Afterwards, the solvent is removed by suitable physical treatment (e.g., evaporation) [64].
2. Supercritical fluid technique: Supercritical fluids are dense as liquids but have viscosity as low as that of gas. In this technique, a solution of the drug efficiently swells the matrix; the solvent is then easily removed by decreasing the pressure, leaving the drug behind in the matrix [65].
3. Direct compression method: In this method, the drug is grounded and mixed with the matrix and a binder in a definite proportion in the presence of a volatile solvent. Next they are compressed into tablets under high pressure (2–3 tons/cm^2) [66].

Direct compression method is the simplest and most economically viable method of tablet preparation.

15.2.5 Concept of Inter-Polymer Network (IPN)

The controlled and targeted drug delivery by inter-polymer networking system has been widely used for drug formulation. An inter-polymer network is a crosslinked polymer, a combination of two different types of polymeric systems which are not covalently linked to each other [67]. The IUPAC definition states that the IPN is "a polymer comprising two or more networks which are at least partially interlaced on a molecular scale but not covalently bonded to each other and cannot be separated unless chemical bonds are broken." A mixture of two or more preformed polymer networks is not an IPN [68]. The physical property of two different polymers are almost retained in the same phase because the infinite zero viscosity gel is formed in the reaction mixture. The IPN is formed when a hydrophilic polymer is interpenetrated with a relatively hydrophobic polymer. This is expected to improve the capability of immobilizing drug. Based on the arrangement pattern the IPN is classified into four types:

1. Sequential IPN: In a sequential IPN system the second polymeric component is polymerized after the completion of polymerization of the first polymeric component [69].
2. Novel IPN: A novel IPN system consists of two or more polymeric networks which are partially interlocked to each other but not covalently linked [69].
3. Semi IPN: In a semi IPN system one of the polymeric components is crosslinked and another is in linear form [69].
4. Simultaneously IPN: Simultaneously IPN system is formed by the simultaneous polymerization of both components of polymer [69].

15.2.5.1 Synthetic Pathway of IPN

The IPNs are prepared by two main synthetic pathways:

1. *In-situ* synthesis: All of the reactants are mixed before the triggering of any polymerization reaction. The syntheses of the two networks (IPN case) may or may not be initiated at the same time, leading either to the simultaneous or the sequential formation of the networks. Hence, the reaction mechanisms leading to the formation of each network must be different in nature, otherwise a single copolymer network (bicomponent network or co-network) would be built-up through crossed reactions [70]. In the case of this *in-situ* synthetic pathway, the obtained material morphology can be modulated almost at will, and made highly different by altering the proportions of the two partner polymers, the order, and/or the relative rates of formation of the two networks [71,72]. This is shown in Figure 15.3.
2. Sequential synthesis: A polymeric network is synthesized and subsequently swollen with all the precursors necessary for the formation of

the second network, which is then carried out within the first network. In this process, the first network generally determines the morphology of the final material. In addition, the composition range is limited by the maximum swelling capacity of the first network by the second network of precursors. It is important to note that the pore-filling electrolyte membranes can be considered as IPNs depending on whether or not the polymer substrate is crosslinked. Indeed, the pores of a porous polymer substrate are filled with polyelectrolyte precursors and the linear or network polyelectrolyte is synthesized within the porous substrate [69]. This is shown in Figure 15.4.

Water-in-oil (w/o) emulsion-crosslinking method is a very common method for the synthesis of IPN hydro gel by sequential pathway. Figure 15.5 is a schematic representation of stearic acid-coated IPN microsphere prepared by water-in-oil (w/o) emulsion-crosslinking method [73].

15.2.6 '*In-Vitro*' Drug Release Study

The oral mode of taking drugs has been quite prevalent and popular for ages. The basis of carrying out drug dissolution tests is to simulate the gastrointestinal tract, where

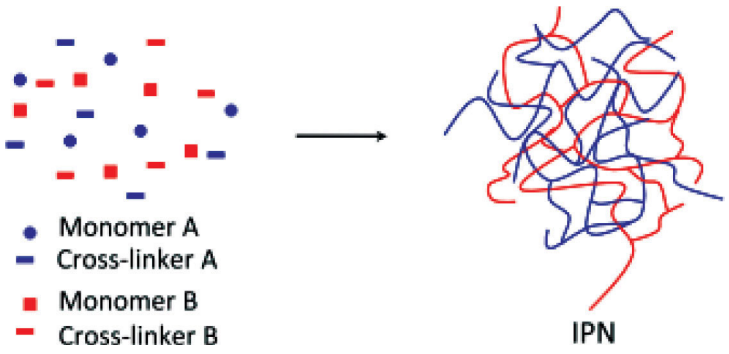

Figure 15.3 Schematic representation of *in-situ* synthesis pathway of IPN [69].

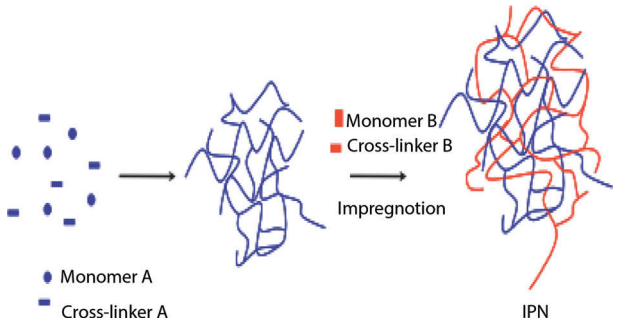

Figure 15.4 Schematic representation of Sequential synthesis pathway of IPN [69].

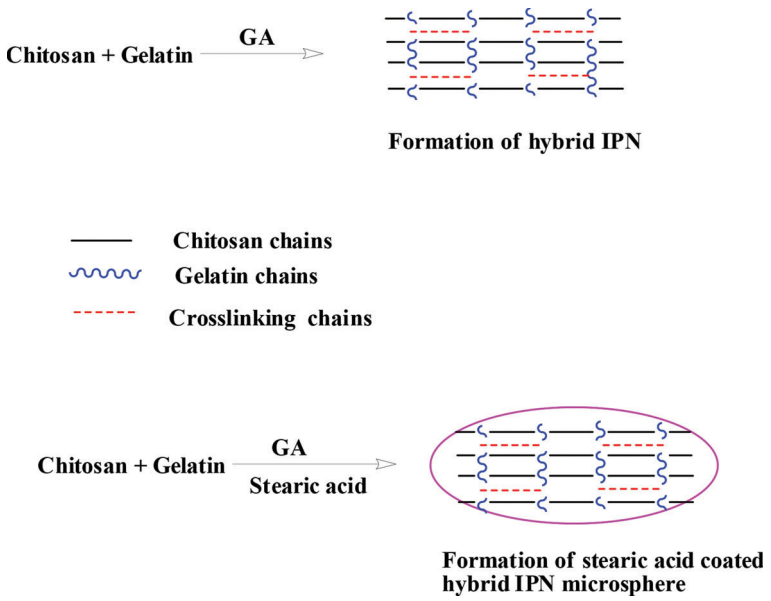

Figure 15.5 Schematic representation of stearic acid-coated IPN microsphere [73].

it is released from the matrix or microsphere and dissolved in aqueous-based gastrointestinal tract fluid. Being a critical part of drug formulation, all aspects of '*in-vitro*' drug dissolution studies have been extensively standardized by USP guidelines (general chapter <711> dissolution) [74].

In fact, dissolution testing is basically a specific form of solubility testing. However, it differs from the latter by the fact that here the measurements are taken multiple times, usually below saturation at physiological temperature (37°C), whereas solubility is measured at standard room temperature to account for local environmental conditions.

Drug dissolution testing for oral mode formulations are performed in various buffers representing the pH of various regions of the gastrointestinal tract. The tests are conducted in paddle/basket-type standard USP drug dissolution rate test apparatus. The pot contains 900 ml of the buffer solution maintained at 37°C resembling body temperature (using isothermal bath). The rotation is maintained between 50–150 rpm. Aliquots are drawn at equal intervals of time, drug content assayed and expressed in form of drug release profile (plot of cumulative drug release (%) vs time).

The real challenge in operating these systems lies in obtaining reproducible results and co-relating them to corresponding biological responses (e.g., bioavailability of the drug, etc.), i.e., *in-vitro/in-vivo* correlation [75].

15.2.7 Mechanism of Drug Release

The mechanism of drug release is dependent on the nature of the matrix and on the mode of drug delivery. For example, diffusion of drug through skin is the mechanism

involved in the case of transdermal drug release. The mechanism of drug release involves one or more of the following:

1. Diffusion
2. Erosion
3. Osmosis

The drug release kinetics from the different matrices is determined by this empirical equation (commonly referred as power-law expression) [76]:

$$M_t/M_\infty = Kt^n \tag{15.2}$$

The value of exponential component 'n' indicates the mechanism of drug transport from the tablet, where M_t and M_∞ represent the amount of solvent diffused into the matrix at time *t* and at infinite time (equilibrium state), respectively,'*k*' is a constant representing the apparent release rate (%/min) that takes into account the structural and geometric characteristics of the release device, and '*n*' is the diffusion exponent. The value of '*n*' is useful for the determination of drug release mechanism. This equation must hold only for the first 60% of the fractional drug release from the tablets ($M_t/M_\infty \leq 0.6$; $\log(M_t/M_\infty) \leq -0.22$), for which the one-dimensional diffusion under a perfect sink condition holds true [76.77].

In the case of non-swelling tablets, drug release is generally expressed by Fickian diffusion, for which $n = 0.5$. For most erodible matrices, the drug release follows zero order kinetics, for which $n = 1$. In the case of swelling tablets, the drug release is due to a combination of swelling and erosion. They follow non-Fickian release behavior. For them, the value of '*n*' lies between 0.5 and 1.0 [78]. The value of '*n*' was calculated from the slopes of the $\log(M_t/M_\infty)$ vs log *t* plot in acidic and neutral pH.

$$\log\left(\frac{M_t}{M_\infty}\right) = \log k + n\log t \tag{15.3}$$

Usha Rani *et al.* reported the polyacrylamide grafted Agar matrix for controlled release of 5-amino salicylic acid (5-ASA). From the results we see that the rate of the drug release will increases with increasing pH of the dissolution medium. The drug release maximum at pH = 7 and drug release is minimum at pH = 2. The drug release in a controlled way also depends upon the percentage of grafting of the polysaccharide [79]. This is shown in Figure 15.6. Pal *et al.* reported the carboxymethyl tamarind (CMT) from tamarind kernel polysaccharide (TKP) matrix for drug delivery agent [80]. Sen *et al.* reported the polyacrylamide grafted carboxymethyl starch (CMS-g-PAM) matrix for sustained drug release of 5-amino salicylic acid (5-ASA) [81].

To describe the drug release kinetics from inter-polymeric networking systems, various types of empirical equations have been developed such as zero-order rate equation, which describes the inter-polymeric systems where the release rate of drug is independent of concentration of the dissolved species. The first-order equation states that the

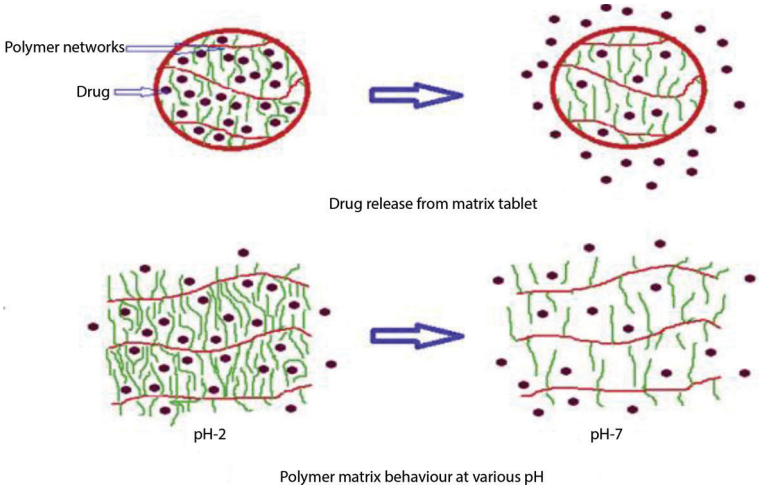

Figure 15.6 Schematic representation of drug release mechanism from matrix tablet at various pHs [79].

drug dissolution rate from the system is dependent on the concentration of dissolved species. The Higuchi square root equation states that the drug dissolution rate depends upon the rate of drug diffusion. The Hixson-Crowell cube root equation states that the drug dissolution rate depends upon the change of surface area of the systems and diameter of the partials [73].

The zero-order equation is:

$$Q = Q_0 - K_0 t \tag{15.4}$$

where Q is the amount of drug present in the system at time t, Q_0 is the amount of drug present in the system at time t = 0, K_0 is the zero-order rate constant.

The first-order equation is:

$$\text{Ln } Q = \ln Q_0 K_1 t \tag{15.5}$$

where K_1 is the first-order rate constant.

The Higuchi square root equation is:

$$M_t = K_H t^{1/2} \tag{15.6}$$

where M_t is the amount of drug release of the system, K_H is the Higuchi rate constant.

The Hixson-Crowell cube root equation is:

$$Q^{1/3} = Q_0^{1/3} - K_c t \tag{15.7}$$

where K_c is the cube root law release constant.

For the determination of n and k value of the system the empirical equation is used:

$$M_t / M_\infty = K t^n \tag{15.8}$$

where M_t/M_∞ is the fraction of drug release at time t, k is the rate constant which represents the drug polymer interaction, n is the emperical parameter that represents the nature of the drug release mechanism of the system. For the Fickian diffusion of system, the value of n is 0.5 and for non-Fickian diffusion the n value is higher than 0.5 but less than 1 [73].

Angadi *et al.* have reported the stearic acid-coated chitosan-based interpenetrating polymer network for controlled release of an antituberculosis drug [73]. Kaity *et al.* have reported the carboxymethylation of locust bean gum in a interpenetrating network for controlled release of buflomedil hydrochloride [67].

15.3 Conclusions

Among the grafted polymeric materials, grafted polysaccharides are generally used for drug formulation because of their nontoxic nature, easy availability and low cost. The drug formulation research in the pharmaceutical industries is dependent upon the polymeric grafted materials because of their eco-friendly nature and other advantages. The matrix and inter-polymeric networking systems are widely applicable methods for controlled and targeted drug delivery systems. Further research is going on to develop the matrix and inter-polymeric networking system.

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16

Pharmaceutical Natural Polymers: Structure and Chemistry

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Abstract
For decades, a wide range of polymeric materials have been used in pharmaceutical technology. Modern drug delivery systems based on biocompatible and biodegradable polymers are the result of the rapid evolution of some highly topical research areas, including molecular biology, nano(bio)technology, nanomedicine, pharmacogenomics, proteomics, bioinformatics, and *in-silico* technology. The manufacturing of different biomaterials applied for targeted, sustained and controlled release of drugs, and for tissue engineering, is also based on the natural, semisynthetic or synthetic polymers. Starch, dextran, cellulose, pectins, alginates, agar, gums, chitosan, hyaluronic acid, collagen, and gelatin are the main natural nontoxic polymers, obtained through sustainable, simple and low-cost methods. In addition, during the last 25 years, the tremendous development of polymer engineering has allowed the use of some semisynthetic and synthetic materials for drug delivery, such as modified starch, cyclodextrins, cellulose, alginate, scleroglucan and chitosan derivatives, and poly(ethylene glycols), polyvinyl alcohol, polyvinylpyrrolidone, polyacrylates, polylactic acid copolymers, ethylene–vinyl alcohol, ethylene–vinyl acetate, ethylene–acrylic acid copolymers, polyethoxylated sorbitans and carbopols, respectively. Therefore, the research in the field of polymeric materials is focused on the drug industry mainly for the optimization of bioavailability, controlled release of active molecules, new ways and new routes of administration, and development of novel pharmacological agents with clinical uses.

Keywords: Natural polymers, biocompatible, biodegradable, pharmaceutical technology, drug delivery

16.1 Introduction

Due to their importance as excipients for the formulation of pharmaceutical dosage forms, optimization of drug delivery and development of novel pharmacological

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agents, polymeric materials are essential for the drug industry: e.g., for biodegradable polymeric nanoparticles [1,2], biomimetic polymers [3], polymeric vesicles for drug and gene delivery [4], nonwoven polymeric nanofibers as novel carriers for the delivery of therapeutic molecules [5], noninvasive insulin delivery systems for the effective treatment of diabetes [6].

Natural biocompatible and biodegradable macromolecules, especially plant-derived biopolymers, are more accessible, eco-friendly and cost-effective as compared with synthetic polymers [7–11]. Thus, starch, dextran, cellulose, pectins, alginates, agar, gums, chitosan, hyaluronic acid, collagen, and gelatin are viable alternatives to synthetic polymers in pharmaceutical technology [12–16].

Another advantage is that natural polymers can be modified using suitable reagents to obtain semisynthetic polymers. Modified starch/dextran, cyclodextrins, cellulose, alginate, scleroglucan and chitosan derivatives are the most important semisynthetic polymers for the drug industry [17–19]. In this respect, starting from the natural cellulose, at least 10 semisynthetic derivatives are widely used for the optimization of pharmaceutical formulations: cellulose acetate (CA), cellulose acetate butyrate (CAB), cellulose acetate phthalate (CAP), methylcellulose (MC), ethylcellulose (EC), hydroxyethyl cellulose (HEC), hydroxypropyl cellulose (HPC), hydroxypropyl methylcellulose (HPMC), carboxymethylcellulose (CMC), nitrocellulose [16,20,21].

A wide range of synthetic polymers are used in pharmaceutical technology. Of these, the most important are poly(ethylene oxide) (PEO) and copolymers, poly(ethylene glycols) (PEGs) and copolymers – PEG–poly(vinyl alcohol) (PVA) and PEG–poly(propylene glycol)–PEG (Pluronic), polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), polyacrylates – polyacrylic acid (PAA), poly(methyl methacrylate) (PMMA)-based copolymers (Eudragit) and poly(butyl cyanoacrylate) (PBCA), polylactic acid (PLA) copolymers – poly(lactic-co-glycolic acid) (PLGA) and PLA–PEG, ethylene–vinyl alcohol (EVOH), ethylene–vinyl acetate (EVA), ethylene–acrylic acid (EAA) copolymers, polyethoxylated sorbitans (polysorbates, tweens) and carbopols [16,22,23]. Drug polymer conjugates, block copolymers, hydrogels and certain water-soluble drug-polymer complexes are also very important for pharmaceutical technology [24].

In the last decade, modern research has highlighted the importance of novel formulations for drug delivery systems, including biodegradable polymeric nanoparticles based on PLGA, PLA, chitosan, gelatin, polycaprolactone and poly-alkyl-cyanoacrylates for nanoencapsulated drugs [25], pharmaceutical polymeric nanoparticles prepared by the double (multiple)-emulsion solvent evaporation technique [26], liposomes, proliposomes, microspheres, gels, prodrugs and cyclodextrins for parenteral and nasal delivery of peptides and proteins [27], interpenetrating polymer networks (IPNs) polysaccharide (alginate and hialuronic acid) hydrogels for drug delivery and tissue engineering [28,29], and magnetic microspheres as novel drug delivery systems [30].

16.2 Natural Polymers

16.2.1 Polysaccharides

Due to their useful properties, natural polysaccharides are components of great importance in the pharmaceutical industry for the formulation and optimization of drug

delivery systems [31]. They are nontoxic, biocompatible, biodegradable, environmentally friendly, less expensive and widely available polymers. It is known that natural gums and mucilages are widely used for conventional and novel pharmaceutical dosage forms [32–36]. In fact, natural polysaccharides are versatile excipients used for the design of controlled drug delivery systems, such as polysaccharide-based micelles for the delivery of hydrophobic molecules and proteins [37–39], formulation and evaluation of pediatric azithromycin suspension using natural gums [40], design and development of atenolol matrix tablet employing natural polymers (starch, xanthan gum, vee gum, guar gum, Arabic gum, tragacanth, hupu gum) [41], formulation of binary mixture tablets of papain, microcrystalline cellulose (MCC), dicalcium phosphate dihydrate (DCP), carrageenan, tragacanth, and agar, prepared by direct compression [42].

16.2.1.1 Starch (Amylum)

Pharmaceutical starch, a homoglycan containing 15–25% amilose and 75–85% amilopectin, is obtained mainly from wheat (*Triticum aestivum*), maize (*Zea mays*) and rice (*Oryza sativa*) grains, and also from potato (*Solanum tuberosum*) tubers or tropical roots [43–45]. Amilose-rich, pregelatinized starches are widely used as excipients for microspheres, granules, inert spheres, hydrophilic matrices, coating compositions: pregelatinized maize starch compared with maize starch as ingredient in pharmaceutical solid dosage forms [46]; pregelatinized starch disintegrants, corn starch, *Carica papaya* and *Curcuma angustifolia* starches as pharmaceutical excipients for paracetamol (500 mg) tablets [47–49]; pregelatinized potato starch as excipient for controlled release of paracetamol, lidocaine and procaine hydrochloride directly-compressible tablet formulations [50]; phosphate-modified and pregelatinized sweet potato starches as disintegrants for paracetamol tablet formulations [51]; porous starch-based drug delivery systems processed by a microwave route for non-steroidal anti-inflammatory drugs (NSAIDs) [52]; the use of pregelatinized starch to develop powder-filled hard capsules of theophylline [53]; multifunctional drug delivery systems (DDS) using starch-alginate beads for controlled release of L-phenylalanine [54]; amylase–ethylcellulose films cast from organic-based solvents as potential coatings for colonic drug delivery [55,56]; coated starch capsules for site-specific drug delivery into the lower gastrointestinal tract (TARGIT technology) – application of pH-sensitive coatings onto injection-molded starch capsules for the treatment of inflammatory bowel diseases [57]; starch-based biodegradable hydrogels with potential biomedical applications as DDS [58]; coating films prepared with chayote tuber and potato starches blending with cellulose nanoparticles [59]; potato starch-blended alginate beads for prolonged release of tolbutamide [60]; preparation and pharmaceutical evaluation of new sustained-release capsule including starch-sponge matrix (SSM) and model drugs (uranine, indomethacin, nifedipine) with different physicochemical properties [61].

16.2.1.2 Dextran

Through microbial-controlled biosynthesis, sucrose is converted into dextrans by immobilized cells of *Leuconostoc mesenteroides* (*Micrococcaceae*) B512 strains. Dextran is used in different pharmaceutical formulations: sugar cane native dextran as an innovative functional excipient for the development of pharmaceutical tablets containing

commercial excipients (lactose, cetyl alcohol, HPMC) and drugs (propranolol hydrochloride, acetyl salicyclic acid, isosorbide dinitrate, lobenzarit disodium, nifedipine) [62]; fast-dissolving maltodextrin applied for the design of oral fast-dissolving films [63]; bio(muco)adhesive properties of β-limit dextrin, which can be digested by salivary amylase and thus provide a clean mouth feel [64]; design and evaluation of oral sustained release formulations based on dextran with theophylline [65]; development and characterization of water-soluble dextran–fatty acid esters as excipients for colon targeting [66]; low molecular weight dextrans stabilize non-viral vectors during lyophilization at low osmolalities—concentrating suspensions by rehydration to reduced volumes [67]; dextran as an excipient for formulating inhaled PYY3-36 peptides and insulin formulations [68].

16.2.1.3 Cellulose

Microcrystalline cellulose is obtained from especially agricultural byproducts (corn cob, sugar cane bagasse, rice husk, cotton) through acid hydrolysis, neutralization, clarification, and drying [69]. A recent study evaluated bacterial cellulose produced form *Acetobacter xylinum* as a pharmaceutical excipient for tablet formulations [70]. Due to its good flowability, compressibility and compactibility, microcrystalline cellulose (MCC) is widely used as an excipient for direct compression (granules, inert spheres, hydrophilic matrices): effect of compressional force on the crystallinity of directly compressible cellulose excipients – low crystallinity cellulose (LCPC), microcrystalline celluloses (Avicel PH-101, PH-102 and PH-302 grades), and powdered cellulose (Solka Floc BW-100) [71]; powdered cellulose as excipient for extrusion/spheronization pellets of a cohesive hydrophobic drug furosemide [72]; combined effects of wetting, drying, and microcrystalline cellulose type on the mechanical strength and disintegration of pellets [73]; cellactose, a co-processed excipient obtained from microcrystalline cellulose co-processed with alpha-lactose monohydrate [74]; mechanistic analysis of drug release from theophylline pellets coated by films containing pectin, chitosan and Eudragit RS [75]; the use of nanocrystalline cellulose for the binding and controlled release of ionizable drugs tetracycline and doxorubicin and hydrophobic anticancer drugs docetaxel, paclitaxel, and etoposide [76]; spray-dried cellulose nanofibers as novel excipient for paracetamol tablets prepared by direct compression and after wet granulation [77]; pharmaceutical acrylic beads obtained by suspension polymerization containing cellulose nanowhiskers as excipient for propranolol hydrochloride delivery [78]; co-processed MCC–Eudragit E excipients for extrusion–spheronization of pellets, incorporating higher amounts of sorbitol (50%) and exhibiting a very high dissolution rate of hydrochlorothiazide and a rapid disintegration in the dissolution medium [79].

16.2.1.4 Hemicellulose: Xyloglucan

Xyloglucan, a hemicellulose obtained from the primary cell wall of vascular plants [80], has certain pharmaceutical applications as excipient for different dosage forms: cysteine–xyloglucan conjugate as mucoadhesive polymer [81]; *in-situ* gelling xyloglucan/alginate liquid formulation for oral sustained drug (paracetamol) delivery to dysphagic patients [82]; *in-vitro* evaluation of theophylline matrices using xyloglucan extracted

from *Tamarindus indica* [83]; preparation and evaluation of extended release matrix tablets of diltiazem hydrochloride using blends of tamarind xyloglucan with gellan gum and sodium carboxymethyl cellulose [84]; preparation, characterization and pulmonary pharmacokinetics of montelukast–xyloglucan microspheres as dry powder inhalation [85]; optimization of carboxymethyl-xyloglucan-based tramadol hydrochloride matrix tablets using simplex centroid mixture design [86]; design, optimization and *in-vitro* characterization of metformin hydrochloride oral *in-situ* gel [87]; taste masked beads of ondansetron hydrochloride using natural polymer xyloglucan and ionotropic-gelation technique [88].

16.2.1.5 Pectins

Pharmaceutical pectins are heteroglycans extracted mainly from industrial waste of orange/mango/banana fruit peels [89–91]. Pectins are used as excipients in solid dosage forms: pectin-based systems for colon-specific drug delivery via oral route – pectin are degraded only by colonic microflora [92–95]; crosslinking of amidated low-methoxylated pectin with calcium during extrusion/spheronization of spherical pellets [96]; evaluation of banana peel pectin as excipient in solid/semisolid oral dosage forms of diclofenac sodium [97]; formulation and evaluation of ibuprofen tablets using orange peel pectin as binding agent [98]; a novel colon-targeted tablet formulation developed using pectin as carrier and diltiazem hydrochloride and indomethacin as model drugs [99]; chronotherapeutic drug delivery of pectin versus guar gum and xanthan gum controlled-release colon-targeted directly-compressed propranolol hydrochloride matrix tablets [100]; pectin/chitosan/Eudragit RS mixed-film coating for sigmoidal delivery of theophylline pellets [101,102]; phosphated crosslinked pectin as a potential excipient for specific delivery of theophylline [103]; diphasic drug release, permeability and swelling of pectin/ethylcellulose films, and *in-vitro* and *in-vivo* correlation of film-coated pellets in dogs [104]; pharmacokinetic comparisons of three nasal fentanyl formulations based on pectin, chitosan and chitosan–poloxamer 188 [105].

16.2.1.6 Alginates

Alginates (alginic acid, sodium alginate) are heteroglycans containing manuronic acid and guluronic acid, separated and purified from the mucilage of some *Laminaria* spp. (*Phaeophyceae*) brown algae: *L. cloustonii* Edmonston, *L. digitata* (Hudson) J.V. Lamouroux, *L. saccharina* (L.) J.V. Lamouroux, *L. japonica* J.E. Areschoug. Alginates are widely used as pharmaceutical excipients for different emulsions, suspensions, retard and enterosoluble formulations [106,107]: sodium alginate extracted from *Sargassum subrepandum* as alternative binder in tablet formulations [108]; preparation of alginate beads for floating drug delivery systems (FDDS) – effects of CO_2 gas-forming agents [109]; production of alginate microspheres by internal gelation using an emulsification method [110]; characterization of the behavior of alginate-based microcapsules *in vitro* and *in vivo* [111]; drug delivery using alginate beads prepared by ionotropic gelation technique [112]; release characteristics of ibuprofen from excipient-loaded alginate gel beads [113]; modified release chitosan, alginate and Eudragit RS microparticles for non-steroidal anti-inflammatory drugs (NSAIDs) formulations [114]; metal

ion-induced alginate–carob gum inter-penetrated (IPN) microspheres for sustained oral delivery of aceclofenac [115]; effect of various excipients on theophylline-loaded alginate beads prepared by ionic crosslinking method [116]; development of a novel floating *in-situ* gelling system for gastric-specific drug delivery of the narrow absorption window drug baclofen [117]; influence of the preparation procedure and chitosan type on physicochemical properties and phenytoin release behavior of alginate–chitosan microparticles [118]; alginate-based oral drug delivery system/microspheres for the treatment of tuberculosis (isoniazid, rifampicin, pyrazinamide) [119,120]; formulation and optimization of raft-forming chewable tablets containing H2 antagonist famotidine [121]; synthesis of thiolated alginate and evaluation of mucoadhesiveness, cytotoxicity and release retardant properties – matrix tablets based on sodium alginate–cysteine conjugate and native sodium alginate containing tramadol hydrochloride [122]; influence of coating on the triamcinolone release of alginate–chitosan beads for colonic drug delivery [123]; composite alginate hydrogel microparticulate delivery system of zidovudine hydrochloride based on counter ion-induced aggregation [124]; alginate and chitosan as polyionic hydrocolloids for the intestinal delivery of protein drugs [125]; controlled release of *Lactobacillus rhamnosus* biofilm probiotics from alginate–carob gum microcapsules [126].

16.2.1.7 Agar

Agar is the purified mucilage obtained by extraction from different red algae species (*Rhodophyceae*): *Gelidium amansii* (J.V. Lamouroux) J.V. Lamouroux, *G. corneum* (Hudson) J.V. Lamouroux, *G. cartilagineum* (L.) Gaillon, *G. subcostatum* Okamura, *G. japonicum* (Harvey) Okamura, *Eucheuma denticulatum* (Burm.) Collins et Hervey, *Pterocladia capillacea* (Gmel.) Born. Et Thur., *Gracilaria lichenoides* (L.) Harv., *G. confervoides* (L.) Greville, *Phyllophora rubens* (Goodenough et Woodward) Greville. It is widely used as pharmaceutical excipient for emulsions, suspensions, suppositories, tablets and also for modern delivery systems: co-precipitation with PVP and agar to improve physicomechanical properties of ibuprofen [127]; a floating controlled-release based on agar gel matrix for theophylline delivery [128]; design and evaluation of cost-effective orodispersible tablets of diethylcarbamazine citrate by effervescent method (16% w/w treated agar, 15% w/w sodium bicarbonate, 15% w/w tartaric acid) [129]; modified polysaccharides (treated agar, co-grinded agar) as fast disintegrating excipients for orally disintegrating tablets of fexofenadine hydrochloride [130]; metformin hydrochloride-loaded mucoadhesive agar microspheres for sustained release [131]; modified polysaccharides (co-grinded treated agar, co-grinded treated guar gum) as fast disintegrating excipients for orodispersible tablets of roxithromycin [132].

16.2.1.8 Carrageenan

Carrageenan represents the mucilage extracted from two red algae (*Gigartinaceae*): *Chondrus crispus* Stackhouse and *Mastocarpus stellatus* (Stackhouse) Guiry sin. *Gigartina mamillosa* (Goodenough et Woodward) J. Agardh. Of the seven purified galactan-sulphates, the most important are κ- and λ-carrageenans. Carrageenans are used mainly for their emulsifying and suspending properties in different dosage forms:

controlled-release tablet matrices from commercial carrageenans – ι-carrageenan (Gelcarin GP-379), κ-carrageenans (Gelcarin GP-812 NF and GP-911 NF), λ-carrageenan (Viscarin GP-209) and τ–carrageenan –, associated with microcrystalline cellulose [133,134]; carrageenans as excipients for pellets obtained by extrusion/spheronization [135]; κ-carrageenan as alternative pelletization aid to microcrystalline cellulose in extrusion/spheronization technique for the formulation of acetaminophen, theophylline, mesalamine and hydrochlorothiazide pellets [136–138]; development of oral controlled-release tablet formulations based on diltiazem hydrochloride–carrageenan complex [139]; preparation and *in-vitro* evaluation of verapamil hydrochloride and ibuprofen containing carrageenan beads [140]; colposcopic evaluation of a vaginal gel formulation of 2% ι-carrageenan to block mucosal transmission of human immunodeficiency virus (HIV) [141]; design and development of hydrogel nanoparticles based on chitosan and carrageenan for mercaptopurine delivery [142].

16.2.1.9 Pullulan

Pullulan is used as an excipient for different modern pharmaceutical formulations, including: development and optimization of dosage forms for the treatment of copper deficiency – tablets, prepared by a wet granulation technique, containing a Cu(II) complex with polysaccharide pullulan [143]; film-forming polymers (pullulan, HPMC E-15) for oral fast dissolving strips [144]; protein refolding assisted by self-assembled nanogels of cholesterol-bearing pullulan as novel artificial molecular chaperone [145]; a novel multicompartimental system based on aminated PVA microspheres/succinoylated pullulan microspheres for oral delivery of sodium diclofenac [146]; photodynamic therapy of C(60)-fullerene modified with pullulan on hepatoma (HepG2) cells [147]; self-quenching polysaccharide-based nanogels of pullulan/folate-pheophorbide-A (photosensitizer) conjugates for photodynamic therapy [148].

16.2.1.10 Scleroglucan

Scleroglucan, an exopolysaccharide secreted by the fungus *Sclerotium rolfsii* Sacc. (*Basidiomycetae*), exhibited different industrial application as a versatile hydrocolloid [149] for modified drug delivery [150,151]: sustained release oral tablets [152]; two galactomannans (guar gum and carob gum) and scleroglucan as matrices for drug delivery of theophylline, vitamin B_{12} and myoglobin [153]; scleroglucan as a drug delivery vehicle, suspension stabilizer and emulsifier for theophylline gel matrices [154]; a novel hydrogel system based on scleroglucan/borax suitable for delivery of theophylline, vitamin B_{12} and myoglobin [155–157]. In addition, a recent study explored the thickening capacity and proper compatibility of scleroglucan with commercial thickeners (corn starch, gum arabic, CMC, gelatin, xanthan, pectin), glycols (ethylene glycol and PEG), alcohols (methanol, ethanol, 1-propanol, 2-propanol), and polyalcohols (sorbitol, xylitol, mannitol) [158].

16.2.1.11 Other Natural Mucilages

Traditionally used as antiasthmatic, febrifuge, antidysenteric, against measles and smallpox, the nontoxic mucilage from the leaves of baobab, *Adansonia digitata* L. (*Malvaceae*), was evaluated as suspending agent. Its physicochemical and suspending

properties are comparable with sodium carboxymethyl cellulose (Na-CMC) [159]. Also, *A. digitata* (AD) mucilage was investigated as suspending agent for pharmaceutical applications, respectively for 125 mg/5 mL paracetamol pediatric suspensions. Different formulations containing 0.1%, 0.2%, 0.3%, and 0.4% (w/v) AD as suspending agent were obtained and their physicochemical properties such as viscosity (ranged from 71–119 cP), sedimentation volume and ease of redispersion were compared with similar paracetamol formulations using Na-CMC as suspending agent. Irrespective of the suspending agent concentrations, paracetamol pediatric suspension formulations containing AD had a better profile than those of Na-CMC. In addition, the internal phase of paracetamol-AD formulations remained suspended for a sufficient time to assure the release of an accurate and uniform dose of active substance during the treatment [160].

In a recent study, the seed mucilage of *Plantago major* L. (*Plantaginaceae*), broadleaf plantain, was investigated as a rate controlling matrix for sustained release of propranolol hydrochloride. For comparison, HPMC K4M and tragacanth gum were used as references. *P. major* seed mucilage (PMSM) powder had a good compactibility, highlighted by physicochemical parameters such as hardness, tensile strength, and friability of tablets (increased as the concentration of mucilage increased). Drug/mucilage ratio characterized the release of propranolol hydrochloride from PMSM matrices: PMSM formulations showed a release rate comparable to HPMC-containing matrices at a lower drug/polymer ratio (drug/HPMC 2:1); at an equivalent content, PMSM is a better release retardant compared to tragacanth gum. The highest correlation coefficient was achieved with the zero-order model during the kinetic analysis of 1:2 drug/PMSM formulation. A corresponding increase of swelling and decrease of erosion was evidenced with the increase of mucilage proportion in tablets. Differential Scanning Calorimetry (DSC) and Fourier Transform Infrared (FT-IR) spectroscopy highlighted that no formation of drug-PMSM complex or changes in drug crystallinity had occurred [161].

Trigonella foenum-graecum L., fenugreek (*Fabaceae*) mucilage was applied in the formulation and evaluation of ciprofloxacin suspension. Different physicochemical parameters such as pH, sedimentation volume, redispersibility, flow rate, viscosity, flocculation degree, and effect of temperature were evaluated for nine batches (C1–C9) prepared with various amounts (0.5–2%) of suspending agent and propylene glycol. By avoiding the aggregation of particles, the increase of suspending agent viscosity provides the stability of suspension (flocculated state) [162].

16.2.1.12 Gums

16.2.1.12.1 Arabic Gum

Arabic (*Acacia*) gum is the crystalline exsudate obtained from the bark of different *Acacia* spp. (*Fabaceae*): *A. senegal* (L.) Willd., *A. nilotica* (L.) Willd. ex Del. sin. *A. arabica* (Lam.) Willd., *A. seyal* Del., spontaneous and cultivated in Africa (Sudan, Egypt, Senegal). Following oral administration, it has a protective effect on cisplatin-induced nephrotoxicity in rats [163]. In the drug industry, Arabic gum (ArbG) is used mainly as emulsifying excipient [164]: in oral rehydration solutions (ORS), sodium absorption was enhanced by ArbG [165]; an enteral formula containing fish oil, indigestible

oligosaccharides, ArbG and antioxidants acts on plasma and colonic phospholipid fatty acid and prostaglandin profiles in pigs [166]; a water-insoluble drug monolithic osmotic tablet system based on ArbG as an osmotic, suspending and expanding agent [167]; preparation and evaluation of pellets containing ibuprofen and theophylline, using *Acacia* and tragacanth gums by extrusion–spheronization [168]; oral sustained release matrix tablets of chlorzoxazone [169]; formulation and *in-vitro* evaluation of ofloxacin tablets using natural gums (ArbG, tragacanth, xanthan gum) as binders [170].

16.2.1.12.2 Tragacanth Gum

Tragacanth gum is obtained similar to Arabic gum, but in this case from the bark of some *Astragalus* spp. (*Fabaceae*): *A. gummifer* Labill., *A. microcephalus* Willd., *A. verus* Olivier, spontaneous and cultivated in Arabic Peninsula and Central Asia. In the food industry, a mixture of whey protein concentrate and tragacanth gum (TrG) may be used as fat replacer in non-fat yogurt [171]. In pharmaceutical formulations, TrG is used as excipient with emulsifying, stabilizing and disintegrant properties. Two recent studies highlighted the optimization of crosslinked tragacanth and comparison of drug release rate profile with synthetic superdisintegrants on metoclopramide orodispersible tablets [172] and the compressibility of different binary mixtures of alpha-amylase and microcrystalline cellulose [173].

16.2.1.12.3 Gellan Gum

Gellan gum, a water-soluble polysaccharide containing D-glucose, L-rhamnose and D-glucuronic acid, is biosynthesized by bacterium *Pseudomonas elodea* (*Sphingomonadaceae*). Different formulations of modern drug delivery systems are obtained starting from gellan gum (GelG): *in-situ* gel systems based on GelG and sodium alginate for the oral delivery of ibuprofen and its pharmacokinetics study in Beagle dogs [174]; development of novel interpenetrating network (IPN) GelG-PVA hydrogel microspheres for the controlled release of carvedilol [175]; huperzine A nasal *in-situ* gel – evaluation of its brain targeting following intranasal administration [176]; ion-activated *in-situ* gel systems of scopolamine hydrobromide – evaluation of its antimotion sickness efficacy [177]; formulation, development and evaluation of patient-friendly dosage forms of metformin based on GelG hydrophilic polymer [178]; ion- and pH-activated novel *in-situ* gel system (chitosan, GelG) for sustained ocular drug delivery of timolol maleate [179]; preparation and evaluation of sublingual tablets of zolmitriptan, a 5-HT receptor agonist (1B/1D) [180]; GelG-based mucoadhesive microspheres of almotriptan malate for intranasal administration in the treatment of migraine – formulation optimization using factorial design, characterization, and *in-vitro* evaluation [181]; L-carnosine as multifunctional dipeptide buffer for sustained-duration topical ophthalmic formulations containing GelG [182]; GelG blended branched polyethylenimine (PEI, 25 kDa) nanocomposites as gene delivery agents [183].

16.2.1.12.4 Ghatti Gum

Ghatti gum (or Indian gum), an arabinogalactan exsudate from the bark of *Anogeissus latifolia* (Roxb. ex DC.) Wall. ex Guill. & Perr., axlewood (*Combretaceae*) [184,185], has potential uses as excipient for different drug formulations due to its properly emulsifying and thickening properties [186].

16.2.1.12.5 Galactomannans: Guar Gum

Guar gum, obtained by processing the endosperm of *Cyamopsis tetragonolobus* (L.) Taub (*Fabaceae*), is a galactomannan containing mainly β-D-mannose and α-D-galactose [187]. Guar gum (GG) and other galactomannas [188] are used as colon-targeting excipients for some drug delivery systems: optimization of compressed GG-based matrix system and influence of formulation on the release rate of drugs (pseudoephedrine hydrochloride, metformin hydrochloride, tetracycline hydrochloride, diclofenac sodium) [189]; purified guar galactomannan as an improved pharmaceutical excipient for the sustained release of a water-soluble drug (ranitidine hydrochloride) [190]; formulation and *in-vitro* evaluation of controlled release matrix tablets of metoclopramide hydrochloride – influence of fillers (lactose monohydrate, microcrystalline cellulose, dibasic calcium phosphate) on hydrophilic natural GG [191]; development of natural gum (Arabic gum, tragacanth, GG) based fast disintegrating tablets of glipizide [192]; preparation and characterization of mucoadhesive microcapsules of gliclazide with natural gums (kondagogu gum, GG, xanthan gum) [193]; paradoxical effect of coating on natural GG blended carbomer matrix systems for neurological depressive disorders (duloxetine hydrochloride) [194]; formulation and evaluation of sustained release matrix tablets of quetiapine fumarate, using GG, tara gum, microcrystalline cellulose (Avicel PH 101), dicalcium phosphate [195].

16.2.1.12.6 Xanthan Gum

Xanthan gum (XG), the exudate biosynthesized by bacterium *Xanthomonas campestris* (*Xanthomonadaceae*), is similar to cellulose. It has different applications in the drug industry for modern dosage forms: XG-based sustained release matrix tablets of diclofenac sodium with microcrystalline cellulose as diluent [196]; formulation and evaluation of once-daily sustained release matrix tablets of aceclofenac using natural gums (XG and karaya gum) [197]; formulation of indomethacin emulgel using two types of gelling agents (XG, Carbopol 934) [198]; modification of palm kernel oil esters nanoemulsions with hydrocolloid XG for enhanced topical delivery of ibuprofen [199]; formulation and evaluation of XG-based sustained release matrix tablets of flurbiprofen using response surface methodology and avoiding the gastric effects of the active principle [200]; the use of hydrophilic natural gums (XG, guar gum) for the formulation of sustained-release matrix tablets of highly water-soluble tramadol hydrochloride [201] and for the formulation and *in-vitro* evaluation of didanosine sustained release matrix tablets [202]; comparison of hydrophilic natural gums (XG, guar gum) and cellulosic polymers (HPMC, CMC) in the formulation of sustained-release matrix tablets of terbutalin sulfate [203]; effect of gums and excipients (XG, guar gum, κ-carrageenan) on drug release and swelling of ambroxol hydrochloride sustained release matrices [204,205]; formulation and evaluation of sustained release matrix tablets of levofloxacin using natural polymers (XG, guar gum, karaya gum) [206]; formulation and evaluation of antipsoriatic gel using natural excipients (XG, egg albumin) [207]; effect of formulation variables on the swelling index of acyclovir sustained release tablets using xanthan gum and sodium alginate [208]; effects of xanthan–carob gum mixtures on the physicochemical properties and oxidative stability of whey protein stabilized oil-in-water emulsions [209].

16.2.1.12.7 Other Natural Gums

16.2.1.12.7.1 Aegle Gum

The purified gum from unripe fruits of *Aegle marmelos* (L.) Corrêa, bael, Bengal quince (*Rutaceae*), is used in different pre-disperse systems for pharmaceutical and food formulations. After freeze drying, the gum particles exhibited optimal excipient properties such as rough surface, moderate negative charge (-16.7 mV), fair flow (repose angle 29–37.2°), moderate compressibility (Carr's Index 17.65%), bulk density (0.42 g/cm^3), tapped density (0.45 g/cm^3), high swelling index (4.2), emulsion stability and capacity better than Arabic gum. In order to modify drug release profile, a high degree of substitution (7.4) allows derivatization or interaction of *A. marmelos* gum (AMG) with different polymers [210]. Six mucoadhesive tablet formulations were obtained by direct compression of diclofenac sodium (as a model drug) with different proportions of AMG (as a binder/sustained-release matrix): 0.25%, 0.5%, 0.75%, 1%, 1.25% and 1.5% (w/w). Then, tablets were evaluated in terms of weight uniformity, hardness, friability, swelling behavior, mucoadhesivity, tensile strength, drug content uniformity and release rate. Optimized formulation exhibited 0.27 N tensile strength, 10 hours of mucoadhesion and complete *in-vitro* release of diclofenac sodium [211]. AMG was also evaluated for its binding properties in the formulation of labetalol hydrochloride tablets obtained by wet granulation technique. Compared to the reference Arabic gum, at the same concentration (5%, w/w), AMG exhibited more optimum results as natural binder for the preparation of uncoated tablet dosage forms [212].

16.2.1.12.7.2 Araucaria Gum

Gel-forming gums extracted from the bark exudates of some *Araucaria* species (*Araucariaceae*) – *A. bidwillii* Hook., bunya pine and *A. heterophylla* (Salisb.) Franco, star pine – are used as pharmaceutical excipients due to their swelling capacity and gelatinization [213,214]. *A. bidwillii* gum is used as a sustained-release matrix in the formulation of diclofenac sodium tablets. Using FT-IR technique, there is no evidence of chemical interaction between drug and gum. By wet granulation method, six formulations were obtained with 10%, 20%, and 30% (w/w) of natural gum powder, 10%, 20%, and 30% (w/w) of HPMC K4M, polyvinyl pyrrolidone (PVP K30) as granulating agent, Avicel pH101 (diluent), magnesium stearate (lubricant) and Aerosil (glidant). Compared to synthetic polymer HPMC K4M, *A. bidwillii* gum (30%, w/w) increased diclofenac sodium release through diffusion mechanism [213].

16.2.1.12.7.3 *Bombax* Gum

Using wet granulation method, the gum from the calyxes of West African trees *Bombax buonopozense* P. Beauv. and *B. ceiba* L. (*Malvaceae*), gold coast bombax, was applied and characterized as an excipient (binding agent) in formulation of immediate-release dosage forms (paracetamol tablets). Compared to tragacanth and *Acacia* gum standards, granules prepared with *Bombax* gum exhibited good flow and optimal compressible properties: repose angle 28.6°, Carr's compressibility 21.3%, Hausner's quotient 1.27. After one hour, hard tablets did not disintegrate and only 52.5% of paracetamol was released with a profile that followed zero-order kinetics. Therefore, *Bombax* gum can be used in the drug industry for controlled delivery at a constant rate over a prolonged period [215,216].

16.2.1.12.7.4 *Cordia Gum*

The gum extracted from fresh and raw fruits of *Cordia obliqua* Willd. sin. *C. myxa* L., clammy cherry (*Boraginaceae*) tree was used in a nontoxic and safe pharmaceutical excipient used as binding agent in different tablet formulations for sustained drug delivery. Diclofenac sodium was used as model drug and *Cordia* gum as novel enteric-resistant sustained-release matrix-forming material [217,218]. Prepared using surface polymerization technique, natural gum microcapsules with/without film-forming material (chitosan) were subsequently compressed into tablets. By analyzing the physicochemical properties of tablets, it was found that *Cordia* gum may be used for microencapsulation and highlighted enteric-resistant sustained-release properties: particle size distribution (volume-weighted mean diameter) 94–780 μm; specific surface area 0.06–0.174 m^2/g; *in-vitro* drug release followed first-order kinetics with $t_{1/2}$ more than 12 hours; in the first two hours, drug release from microcapsule-compressed tablets was resistant in simulated gastric fluid and initiated in the simulated intestinal fluid [219]. In addition, *Cordia* powdered gum mucilage was used for the formulation of oral mucoadhesive chlorhexidine tablets. Mucoadhesive strength of oral tablets containing 20% (w/w) mucilage was significantly higher compared to 30% HPMC. Other pharmacopoeial characteristics of tablets were evaluated as optimal when using natural mucilage compared to HPMC: hardness, friability, disintegration time, dissolution rate (the mean dissolution time – MDT), swelling (the amount of water absorbed by tablets), and mucoadhesive strength (the force needed to separate tablets from mucosa) [220].

16.2.1.12.7.5 Carob (Locust Bean) Gum

Carob (locust bean) gum is a galactomannan-type polysaccharide, a versatile natural polymer with various applications. The natural gum is obtained from the seeds of *Ceratonia siliqua* L., carob tree, locust bean (*Fabaceae*). It is used as an excipient in pharmaceutical technology for different drug delivery systems (thickening, high gelling and stabilizing properties) and for the manufacturing of biocompatible and biodegradable scaffolds in tissue engineering (lack of toxicity). Semisynthetic carboxymethyl-derivatives have been recently developed for the increasing of carob gum aqueous solubility and for the preparation of sustained release microbeads [221–223].

Carob gum (CG) alone and a 1:1 mixture of CG and xanthan gum were used for development of a controlled delivery system for highly water-soluble propranolol hydrochloride. Magnesium stearate and talc (1:2) were added for the increasing of flowability and compressibility of the granules obtained by wet granulation method. The uniform tablet hydration in dissolution (aqueous) media and the precise controlled release of drug are given by the synergistic interaction between the two cost-effective, nontoxic and easily available biopolymers. There is no chemical interaction between drug and natural polymers [224,225].

Other modern research has highlighted the importance of CG for different drug delivery systems, as follows: CG and chitosan combinations (2:3, 3:2, and 4:1) as a carriers for buccal drug delivery of 10 mg propranolol hydrochloride mucoadhesive tablets coated on one face with 5% (w/v) ethyl cellulose or formulated using a direct compression technique [226]; enhancement of solubility, dissolution rate and bioavailability of poorly water-soluble drug lovastatin using modified CG (by heating) and solid dispersion techniques [227]; formulation and evaluation of nimesulide orodispersible tablets

using 10% CG as a natural superdisintegrant against cross-carmellose sodium standard superdisintegrant and marketed nimesulide fast dissolving tablets [228]; development of modern gastroretentive drug delivery system of ziprasidone hydrochloride based on eco-friendly natural gums (CG, okra gum) [229]; improvement of atorvastatin calcium dissolution rate using heat-modified CG and solid dispersion technique (1:6 drug/polymer ratio) [230]; development of sustained release CG–alginate mucoadhesive macromolecules containing aceclofenac through ionotropic-gelation [231].

16.2.1.12.7.6 *Cedrela* Gum

Cedrela gum is a new vegetal product employed as a cheap and effective natural polymer in pharmaceutical formulations. Obtained from the incised trunk of *Cedrela odorata* L., Spanish cedar (*Meliaceae*), the gum was used in sulphamethoxazole suspension and ibuprofen tablet formulations [232,233]. *Cedrela* gum (1–4%, w/w) can replace HPMC and gelatin in sulphamethoxazole suspension, taking into account sedimentation volume, flow rate, viscosity and influence of temperature on these essential parameters [232]. Compared to HPMC, *Cedrela* gum highlighted better compressive, flow and binding properties in ibuprofen tablet formulations obtained through wet granulation method. In addition, it may be employed as bioadhesive excipient and sustained release matrix. Repose angle, Hausner's ratio, Carr's Index, and Heckel, Kawakita and Gurnham plots were used for the assessment of compressional properties. In either phosphate buffer (pH 6.8) or 0.1 M hydrochloric acid environment, bioadhesive evaluation was made by means of rotating cylinder technique. *Cedrela* gum is a low viscosity polymer (48 cPs), significantly more plastic than HPMC, with free hydroxyl groups established by FT-IR spectroscopy. Compared to HPMC binder, tablet formulations containing 2% (w/w) natural gum are non-friable (< 1%), non-disintegrating, with higher crushing strength (130.95 N), better adherence to incised pig ileum, and significantly longer drug release time [233].

16.2.1.12.7.7 *Cissus* Gum

The gum extracted from the stem of *Cissus populnea* Guill. & Perr., a woody African vine from the *Vitaceae* family, has different uses in the drug industry such as dispersant in liquid systems, by its emulsifying and suspending properties [234], and binder in paracetamol tablet formulation [235,236]. Highly flocculated zinc oxide suspensions, exhibiting good redispersibility and stability similar to that prepared with tragacanth mucilage, are achieved at 0.6 to 1% (w/v) *Cissus* gum amounts. Compared to tragacanth and Arabic gum, liquid paraffin emulsion with minimal separation is obtained by *Cissus* gum concentrations above 0.75% (w/v). Globule coalescence and creaming rates were in the order Arabic gum > *Cissus* gum > tragacanth and tragacanth > Arabic gum > *Cissus* gum, respectively [234]. Also, *Cissus* gum can replace gelatin as a better alternative in paracetamol tablet formulations that tend to cap/laminate, mainly for uncoated tablets requiring a rapid drug release. Compared to gelatin excipient, paracetamol tablet formulations based on 2–4% (w/w) *Cissus* gum showed an increase in crushing strength, lamination tendency, plastic deformation during compression and release rate, a decrease in friability, tensile strength and BFI, a faster disintegration and higher CSFR values. However, in paracetamol formulations, compared with *Cissus* gum, gelatin induces higher tensile strength, lower friability, longer disintegration time and a greater tendency to laminate [235,236].

16.2.1.12.7.8 Grewia Gum

Extracted and purified from the inner stem bark of *Grewia mollis* Juss. edible species, *Tiliaceae* family, spontaneously cultivated in Western Africa, grewia gum is used as a thickening/suspending agent (pseudoplastic flow behavior) for the food/cosmetic industry and as mucoadhesive, potential aqueous film coating agent and matrix for controlled drug release in tablets or granulations [237,238]. It contains simple sugars (glucose, galactose, rhamnose, arabinose, xylose) and traces of Ca, Mg, Zn and P, having a good thermal stability [239]. In some cases, grewia gum (GG) can replace and is preferable to Arabic gum or carboxymethylcellulose for different pharmaceutical formulations: ibuprofen suspensions [237]; paracetamol tablets – improving the fluidity of paracetamol granulation better than polyvinylpyrrolidone (PVP) [240]; 40% GG matrix tablets prepared by direct compression for oral controlled delivery of cimetidine, compared with similar formulations based on hydroxypropyl methylcellulose (Methocel), Arabic gum, carboxymethylcellulose (Blanose) and ethylcellulose (Ethocel) as polymer matrices [241].

16.2.1.12.7.9 Hakea Gum

Due to its mucoadhesive and sustained-release properties, hakea gum (HG), obtained from the shrub *Hakea gibbosa* (Sm.) Cav. (*Proteaceae*), is used for the formulation of some buccal tablets containing chlorpheniramine maleate [242,243] or calcitonin [243]. Flat-faced Cutina-coated core tablets containing either 22/32 mg of HG, 25/40 mg of chlorpheniramine maleate and sodium bicarbonate/tartaric acid (1:1.5 molar ratio) were obtained by direct compression technique [243]. Also, an improved transbuccal delivery system for therapeutic polypeptides obtained using direct compression technique supposes flat-faced Cutina-coated core tablets containing either 12/32 mg of HG and 40 μg (200 IU) of salmon calcitonin [244].

16.2.1.12.7.10 Karaya Gum

Similar to other natural biopolymers, karaya gum (*Sterculia* spp. – *S. foetida* L., *Sterculiaceae* family) is used in the pharmaceutical industry mainly as a hydrophilic matrix for controlled drug release [245]: buccoadhesive tablets for sustained release of nicotine containing karaya gum (for its superior mucoadhesive properties), xanthan gum, guar gum or glycol chitosan [246]; sustained-release diltiazem matrix tablets using hydrophilic gum blends, at different ratios of drug/gum (1:1, 1:2, 1:4) – karaya gum alone or in combination with carob gum and hydroxypropyl methylcellulose [247]; metformin hydrochloride floating tablets prepared by wet granulation method with natural gums (karaya, kondagogu) as release retarding polymers [248]; solid mixtures of modified gum karaya and parent gum karaya, prepared by co-grinding technique, as carriers for the dissolution enhancement of poorly water-soluble drug nimodipine [249].

16.2.1.12.7.11 Khaya Gum

In the drug industry, the gum exudate of *Khaya senegalensis* (Desr.) A. Juss., African mahogany (*Meliaceae*) is used for its special properties such as: suspending/emulsifying agent, disintegrant for tablets or hydrogel matrix for modified release dosage forms [250]. The effect of formulation variables, such as granule wetness (binder volume), drying temperature and compression pressure, and the swelling capacity (index) of

the natural polymer were demonstrated using sodium bicarbonate tablets containing *Khaya* gum [251]. Another two studies highlighted the comparative evaluation of the binding properties of two species of *Khaya* (*K. senegalensis* and *K. grandifoliola* C. DC.) gum polymer in a paracetamol tablet formulation [252], and the evaluation of *Khaya* gum as a directly compressible matrix system, against hydroxypropylmethylcellulose (HPMC), for controlled release of paracetamol (water soluble) and indometacin (water insoluble) as model drugs [253].

16.2.1.12.7.12 Kondagogu Gum

The natural excipient kondagogu gum (*Cochlospermum gossypium* DC., Buttercup tree, *Bixaceae* family) has different applications in the pharmaceutical field: as matrix forming carrier for the developing of diltiazem hydrochloride floating tablets, prepared by direct compression technique using PVP K-30 (binder), hydroxypropyl methylcellulose (HPMCK4M) (gel forming polymer) and sodium bicarbonate (CO_2 source) [254,255]; as a natural matrix (mixture of kondagogu and guar gums) for sustained release tablets of 75 mg ambroxol hydrochloride prepared by wet granulation technique [256]; as a drug retarding material in the formulation of antidiabetic gliclazide-loaded pellets, compared with marketed gliclazide, for pharmacodynamic testing in rats [257]; as a biopolymeric carrier for chronotherapeutic (colon specific) delivery of a model opioid drug (tramadol hydrochloride) with analgesic effect in rheumatoid arthritis – core tablets were obtained by wet granulation technique, using 30%, 40%, 50%, 60% and 70% (w/w) natural gum, and then compression coated with Eudragit S100 for the preventing of gastric drug release [258]; as polyelectrolyte complexes of kondagogu gum–chitosan, prepared by mixing polymeric solutions of several concentrations (0.02–0.18%, w/v), for diclofenac delivery [259].

16.2.1.12.7.13 Konjac Gum

Konjac glucomannan (*Amorphophallus konjac* K. Koch, *Araceae* family) and konjac glucomannan–xanthan (1:1) gum mixtures are used as excipients for controlled drug delivery of small molecules, for up to an eight hour period, due to the gel formation [260]. In addition, konjac glucomannan and xanthan gum are recommended as compression coatings for colonic drug delivery of cimetidine tablets [261].

16.2.1.12.7.14 Limonia Gum

A recent study (2013) evidenced that the natural gum isolated from the bark of *Limonia acidissima* L., wood-apple (*Rutaceae*), may be used as release retardant in the formulation and evaluation of sustained-release matrix tablets of nicorandil. All the formulations comply with pharmacopoeial standards. There are no chemical interactions between nicorandil and the natural gum (FT-IR spectroscopy). Physicochemical parameters were found to be optimal for granules (repose angle, loose/tapped bulk density) and compressed tablets (thickness, friability, hardness, drug content, weight variation, *in-vitro* dissolution). Using USP35/NF30 type II dissolution device (50 rpm), it was found that *in-vitro* drug release decreased with increase in gum ratio. The zero-order, first-order model, Higuchi's square-root equation and Korsmeyer-Peppas model applied for the evaluation of the sustained-release kinetics highlighted a good initial release of nicorandil (15% in first hour) and the extension of release for more than 12 hours [262,263].

16.2.1.12.7.15 Mango Gum

In the drug industry, mango gum (*Mangifera indica* L., *Anacardiaceae* family) may be applied for obtaining different uncoated tablet dosage forms [264]. An easily available, safe and low-cost excipient, it was evaluated as a natural binder for paracetamol tablets prepared by wet granulation technique. At 5% (w/w) concentration, the binding efficacy of mango gum is similar to the standard tablet binder Arabic gum [265]. Other studies exhibited the importance of mango gum powder as a granulating and binding excipient in lornoxicam tablets – five different gum concentrations (2, 4, 6, 8 and 10%, w/v), compared with the standard binder starch (10%, w/v) [266], and as a disintegrating agent for metformin hydrochloride mouth dissolving tablets [267].

16.2.1.12.7.16 Odina Gum

Traditionally used in Indian ethnopharmacology, *Odina* gum, extracted from the bark of *Odina wodier* Roxb. (*Anacardiaceae*), has some useful properties in the drug industry: as a tablet binder, most efficient compared with standard 5% starch paste [268]; as an emulsifying agent, producing more stable emulsions at a much lower amount compared with the Arabic gum, with no creaming/cracking even after long storage – obtained by wet gum method, primary emulsion contains 4:2:0.5 oil/water/gum ratio, with Odina gum powder as an emulsifying agent [269]; as a novel matrix-forming material for sustained drug delivery of diclofenac sodium tablets [270,271].

16.2.1.12.7.17 Prunus Gum

Due to its compatibility with active principles and another excipients, *Prunus domestica* (*Rosaceae*) gum was used as a novel tablet binder. Tablets were prepared by wet granulation method under the following experimental conditions: Avicel pH 101 (diluent), *P. domestica* gum (sample binder), PVP K30 (standard binder), magnesium stearate (lubricant), and 10, 15 and 20 mg of sodium diclofenac (model drug) [272]. Also, different studies evidenced the use of *Prunus dulcis* sin. *P. amygdalus* (almond) gum as a pharmaceutical excipient for polysaccharide-based hydrogels [273] and solid oral dosage forms [274], such as diclofenac sodium tablets – with microcrystalline cellulose (diluent), and almond gum and PVP solutions (binders) [275] or almond gum-based colon-targeted tablets of secnidazole and its β-cyclodextrin inclusion complex for the treatment of amoebiasis [276].

16.2.1.12.7.18 Sesamum Gum

Recent studies evaluated *Sesamum indicum* L., sesame (*Pedaliaceae*) gum for its useful properties in the drug industry: as a binder/hydrophilic polymer in the formulation of sustained release paracetamol granules and tablets, compared with standard binders Arabic gum, gelatin, and sodium carboxymethylcellulose [277]; for the intestinal delivery of antifilarial drug diethylcarbamazine tablets [278]; as an accessible, cost-effective and eco-friendly suspending agent in a pediatric pharmaceutical suspensions, compared with Arabic gum and tragacanth [279].

16.2.1.12.7.19 Tamarind Gum

Tamarind (*Tamarindus indica* L., *Fabaceae* family) seed polysachharide/kernel gum was found to be a versatile nontoxic, biodegradable/biocompatible natural polymer: as a drug carrier for some modern bioadhesive and sustained release forms [280–283]; as a binder (tablets) and muco-/bioadhesive matrix for buccal, sublingual, gastrointestinal

and ocular controlled drug delivery [284–288]; as an emulsifying agent (2%, w/v), compared with standard Arabic gum (10%, w/v), for the formulation of castor oil emulsions [289]; as a release modifier for sustained release matrix of diclofenac sodium tablets [290,291], aceclofenac tablets [292], salicylic acid tablets (crosslinked tamarind gum embedded with chemically synthesized ZnS nanocrystals) [293], metformin [294] and famotidine [295] gastro-retentive tablets, prepared by direct compression method.

16.2.1.12.7.20 Terminalia Gum

Also known as bhara gum, the exudate from the incised trunk of some tropical trees of *Terminalia* genus (*T. bellerica* Roxb., *T. catappa* L., *T. randii* Baker f.), *Combretaceae* family, may be used in the formulation of some sustained release pharmaceutical forms: famotidine microcapsules (ionic gelation technique) [296]; dextromethorphan hydrobromide tablets obtained by direct compression, wet granulation and solid dispersion techniques [297]; carvedilol (water insoluble) and theophylline (water soluble) tablets prepared by direct compression – *Terminalia* gum was assessed as controlled release matrix against xanthan gum and hydroxypropyl methylcellulose [298].

16.2.1.13 Aminoglycans:Chitosan

Chitosan is a poly-N-acetyl-glucosaminoglycan prepared by alkaline deacetylation of chitin. It is made of randomly distributed β-(1→4)-D-glucosamine and N-acetyl-D-glucosamine. Produced annually almost as much as cellulose, chitin is the most abundant natural amino polysaccharide, the major component of the exoskeleton of invertebrates, crustaceans and insects [299–301]. In the drug industry, chitosan is a widely used excipient for different formulations with local hemostatic, antibacterial, anti-inflammatory and wound healing effects [302], as a direct tablet compression, tablet disintegrant, for controlled release solid dosage forms and for improvement of drug dissolution [303–306]. Novel drug delivery systems based on chitosan include different formulations: biocompatible and biodegradable chitosan nanoparticles as drug carriers [307]; chitosan–microcrystalline cellulose tablets prepared by direct compressin technique [308]; chitosan beads [112]; chitosan-based gastroretentive floating drug delivery systems [309]; chitosan/Kollicoat SR30D free films for colonic drug delivery, prepared by casting/solvent evaporation method [310]; thermoresponsive/thermosetting hydrogel based on chitosan [311]; diclofenac sodium-loaded chitosan pellets produced by extrusion-spheronization [312]; microcrystalline chitosan (MCCh) as gel-forming excipient in matrix granules for gastric-specific slow-release of ibuprofen, paracetamol and furosemide [313,314]; sustained-release fast-disintegrating multi-unit compressed tablets of lornoxicam containing Eudragit RS coated chitosan-alginate beads [315]; chitosan-based controlled porosity of microbially triggered colon-targeted osmotic pump (MTCT-OP) for colon-specific delivery of budesonide [316]; chitosan and sodium sulfate as excipients in the preparation of prolonged release theophylline tablets [317]; pentoxifylline–chitosan oral matrix tablets prepared by the slugging method [318]; ionic crosslinked chitosan beads for extended release of ciprofloxacin hydrochloride [319]; improved properties of incorporated chitosan film with ethyl cellulose microspheres for controlled release of ciprofloxacin hydrochloride [320]; L(9) orthogonal design-assisted

formulation and evaluation of chitosan-based buccoadhesive films of miconazole nitrate [321]; development and bioadhesive properties of chitosan-ethylcellulose microspheres for nasal delivery of loratadine [322]; chitosan nanoparticles (CS-NPs) for encapsulation and sustained delivery of lenalidomide [323]; permeation-enhancing effects of chitosan formulations on recombinant hirudin-2 (rHV2) by nasal delivery *in vitro* and *in vivo* [324]; chitosan hydrogel as siRNA vector for prolonged gene silencing [325].

16.2.1.14 Glycosaminoglycans:Hyaluronic Acid

Consisting of N-acetyl-D-glucosamine and glucuronic acid monomers, hyaluronic acid (HA) is a naturally occurring non-immunogenic linear polysaccharide. It is used as a pharmacologically active excipient for chemotherapeutic drug formulations, because different tumors exhibited an up-regulated expression/specificity of hyaluronan receptors [326]. In this respect, HA was successfully evaluated as an active excipient for gemcitabine delivery in the non-surgical treatment of pancreatic cancer [327]. Also, different studies highlighted the importance of HA for modern drug delivery systems: spray-dried lipid–hyaluronan–polymethacrylate biocompatible microparticles developed for drug delivery in the peritoneum [328]; mucoadhesive properties of 3:2 mixture of tamarind seed polysaccharide and HA, in aqueous solution, enhanced extra- or intra-ocular drug bioavailability of ketotifen fumarate and diclofenac sodium [329]; HA/chitosan nanoparticles, obtained by ionic gelification technique, as delivery vehicles for pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF-BB) [330].

16.2.2 Peptides and Proteins

Despite their limited oral absorption, because of the intestinal epithelial membrane barrier/degradation, peptides and proteins have been used in the treatment of certain diseases for several decades. Recent studies have exhibited the importance of cell-penetrating peptides (CPPs), such as HIV-1 Tat, penetratin and oligoarginine, for oral (intracellular) delivery of novel biodrug macromolecules [331]. Also, it was demonstrated that biocompatible peptide-coated liposomal fasudil enhances site-specific vasodilation in the treatment of pulmonary arterial hypertension. The liposomes were coated with a cyclic peptide (CARSKNKDC), as a targeting moiety. The mean pulmonary arterial pressure was reduced by 35–40% using inhalable peptide-linked liposomal fasudil, with no influence on the mean systemic arterial pressure [332]. Peptoids, a developing class of peptide-like oligomers, comprise a peptide-based backbone and N-substituted glycines for side-chain residues, ensuring a complete protease-resistance with therapeutic and diagnostic applications [333]. In addition, insoluble and biodegradable whey protein biofilms with suitable mechanical properties and high resistance to proteolytic enzymes are prepared by heat crosslinking/γ-irradiation followed by cellulose entrapping [334].

16.2.2.1 Albumin

For the biopharmaceutical formulation and optimization of matrix hydrolysis, microspheres and nanospheres, different forms of albumin are used in the drug industry

such as human serum albumin (HSA), recombinant human albumin (RHSA), bovine serum albumin (BSA), considering the risk of transmitting viral and prion contaminants. Various studies highlighted the importance of RHSA for drug delivery systems, including aggregation of RHSA, a lyophilized pharmaceutical protein, and the effect of moisture and stabilization by excipients [335,336], freeze drying of RHSA–doxorubicin nanoparticles with different stabilizer excipients (sucrose, trehalose, mannitol) [337], and stabilization and encapsulation of recombinant human erythropoietin (rhEPO) loaded into PLGA microspheres using RHSA as a stabilizer [338]. Also, BSA is used for the formulation of modern pharmaceutical dosage forms, such as non-aqueous encapsulation of excipient-stabilized spray freeze-dried BSA into poly(lactide-co-glycolide) (PLG) microspheres, for the release of native protein [339] and encapsulation of BSA in PLG microspheres by the solid-in-oil-in-water technique [340,341].

16.2.2.2 Casein

Peptides from bovine beta-casein (betaCN), obtained by plasmin hydrolysis and fractionation of the hydrolysate, and crosslinked beta-casein polymers, are used in drug formulation for their emulsion-stabilizing properties [342,343]: crosslinked hydrophilic casein/gelatin beads [344]; network forming properties of various proteins (beta-casein, beta-lactoglobulin, ovalbumin, soy glycinin) adsorbed at the air/water interface in relation to foam stability [345]; development and evaluation of taste-masked drug for pediatric medicines – application to the acetaminophen (bitterness masking) by coating with sodium caseinate and lecithin [346]; preparation (by complex coacervation) and characterization of microcapsules based on biodegradable polymers – pectin/casein complex for controlled drug release of acetaminophen [347]; impact of crosslinking and drying method on the *in-vitro* release of indomethacin or acetaminophen from casein–pectin microparticles [348]; two types of casein hydrolysate, casein A and casein B, as a rapid and/or enteric dissolving additive for oral drugs, such as diclofenac acid, diazepam, and prednisolone [349]; *in vitro–in vivo* evaluation of tableted casein–chitosan microspheres containing diltiazem hydrochloride, prepared using aqueous coacervation technique [350]; improving the stability of insulin in solutions containing intestinal proteases (casein, protamine) *in vitro* [351]; spray-dried respirable powders containing *Myoviridae* bacteriophages for the treatment of pulmonary infections – the phages were incorporated into microparticles based on trehalose, leucine, and optionally a third excipient (a surfactant or casein sodium salt) [352].

16.2.2.3 Collagen

Collagen is used mainly for topical delivery of active principles for wound infections, diabetes, hormonal disorders and for tissue engineering: collagen/PLGA microparticle composites for local controlled delivery of gentamicin, to preserve the positive effect of collagen on tissue regeneration [353]; design and optimization of some collagen-minocycline-based hydrogels potentially applicable for the treatment of cutaneous wound infections [354]; preparation and characterization of marine sponge collagen nanoparticles and employment for the transdermal delivery of 17beta-estradiol-hemihydrate, for hormone replacement therapy [355]; enhanced bioavailability of subcutaneously injected insulin coadministered with collagen in rats and humans, by inhibiting

proteolytic enzymes, mainly collagenase-like peptidase [356]; bone regeneration with recombinant human bone morphogenetic protein-2 (rhBMP-2) using absorbable collagen sponges (ACS) [357,358].

16.2.2.4 Gelatin

Gelatin, a natural polymer derived from collagen, is used for the formulation of biocompatible/biodegradable drug delivery systems (matrix hydrolysis, microspheres, nanospheres) and wound dressings. Due to its film-forming, thermoreversible gelling and adhesive properties, gelatine is an indispensable excipient in the drug industry, mainly for the preparation of soft/hard capsules, as a modern and elegant pharmaceutical dosage form [359] and for vitamin embedding. Different research has exhibited the importance of gelatin for pharmaceutical technology: enteric-coated soft gelatin capsules prepared with hydroxypropyl methylcellulose acetate succinate (HPMCAS) by dry coating technique [360]; porous gelatin capsules of verapamil, prepared by entrapping gas bubbles or by drilling in the capsule wall with syringe needles [361]; crosslinking of gelatin capsules with aldehydes (formaldehyde), with *in vitro–in vivo* enhanced performances [362]; piroxicam-loaded gelatin microcapsules prepared by spray-drying technique [363]; valsartan-loaded gelatin microcapsules without crystal change, using HPMC as a stabilizer and spray-drying technique [364]; gelatin microparticles prepared by co-lyophilization with PEG, as a protein micronization adjuvant [365]; gelatin-based lyophilized orally disintegrating tablets [366]; novel excipient for matrix of diltiazem hydrochloride floating tablets containing nile tilapia gelatin, chitosan and cellulose derivatives (EC, MC, CMC-sodium) [367].

16.2.2.5 Keratin

Keratin represents all intermediate filament-forming proteins from vertebrate epithelia and corneous tissues (horns, claws, hooves). Keratin derivatives are used for different pharmaceutical applications, including chronic wound dressing, drug delivery (controlled release of antibiotics and growth factors), cell and tissue engineering [368]. Using ungula/*stratum corneum* penetration enhancers (e.g., urea, thioglycolic acid, papain), keratin film made of human hair was used as a nail plate model for studying drug permeation [369,370]. Also, keratin sponge/hydrogel II containing porous interpenetrating networks (IPN) was investigated as an active agent for riboflavin delivery into simulated gastric fluid (SGF) [371].

16.2.2.6 Poly-L-Lysine

From the point of view of pharmaceutical technology, poly-L-lysine is used for the optimization of modern ways of administration and drug delivery systems: enhancement of novel transdermal mannitol transport *in vitro* by skin electroporation, using heparin, dextran-sulfate, neutral dextran, and poly-L-lysine [372]; alginate–pectin–poly-L-lysine particulate as a potential controlled release formulation of theophylline, chlorothiazide and indomethacin [373]; drug delivery of oligonucleotides by peptides, including poly-L-lysine alone or in combination with receptor-specific targeting ligands (asialoglycoprotein, galactose, growth factors, transferrin) [374]; extending residence time and stability

of GLP-1 peptide by protected graft copolymer (PGC) excipient – poly-L-lysine was grafted with methoxypolyethyleneglycol and fatty acid at the epsilon amino groups [375]; efficacy of poly-L-lysine, lysine monomer and PEG as excipients for the controlled release of nerve growth factor (NGF) from 2D freeze-dried silk fibroin scaffolds [376].

16.2.3 Resins and Related Compounds

16.2.3.1 Shellac

Different commercially available shellac types, a natural insect resin approved by the Food and Drug Administration (FDA), with certain physicochemical properties, were investigated as free films (ammoniacal solutions) and as micronized powder (acid form) for pharmaceutical applications [377,378]. Also, the effect of alkali treatment on properties of native shellac and stability of hydrolyzed shellac was established [379]. Shellac is used for its coating properties in different formulations, as follows: enteric-coated drug pellets based on aqueous shellac in combination with PVA, HPMC and Carbomer 940 [380]; different aqueous shellac-coated sustained release/colon targeting theophylline pellet formulations containing calcium chloride, citric acid or Eudragit E [381–383]; probiotic formulations based on shellac coatings with improved enteric properties – investigation of the effects of plasticizers (glycerol, glyceryl triacetate) and water-soluble polymers (sodium alginate, HPMC, PVP) on thermodynamic characteristics and coating properties of shellac [384]; paclitaxel-coated balloons, covered with shellac for an uniform distribution of drug at the tissular level, significantly reduce restenosis after percutaneous transluminal angioplasty of the superficial femoral artery [385]; microencapsulated delivery system based on gelatine microspheres for norbormide, a species-specific acute rodenticide, coated with either shellac resin or an equal mixture of shellac and Eudragit RS in a fluid-bed coating machine [386].

16.2.3.2 Rosin (Colophony)

Rosin (colophony), a low molecular weight non-volatile resinous polymer of vegetal origin (*Pinus* spp., *Pinaceae* family), consists mainly of diterpenoid derivatives: levopimaric acid, dextropimaric acid, abietic acid, dehydroabietic acid, hydroabietic acid, pimaric acid, isopimaric acid. It exhibited suitable film-forming properties with applications for targeted drug delivery systems [386–389]: transdermal drug delivery [390]; sustained release rosin microspheres containing aceclofenac microencapsulated using emulsion solvent evaporation technique [391]; rosin-based matrix tablets, prepared by direct compression method, for oral sustained release of diltiazem hydrochloride [392]; natural biodegradable zidovudine (AZT) microcapsules with colophony resin as microencapsulating agent [393].

16.2.3.3 Gum Resins: Copal and Dammar

Because of its film-forming properties, copal resin was used as a coating material for sustained release and colon-targeted drug delivery of diclofenac sodium [394,395] and metformin hydrochloride tablets [396]. Matrix tablets were usually prepared by wet granulation method using 2-propanol as a granulating agent [395]. Dammar is a

natural gum resin obtained from *Shorea javanica* Koord. & Valeton (*Dipterocarpaceae*). Dammar resin as well as copal is used as a novel sustained release matrix-forming excipient in tablet formulation [395,396].

16.2.3.4 Myrrh Oleo Gum Resin

Extracted from the stem of different *Commiphora* spp.—*C. myrrha* (Nees) Engl., *C. habessinica* (O. Berg) Engl., *C. wightii* (Arn.) Bhandari—*Burseraceae* family, myrrh oleo gum resin has been known since ancient times due to its medicinal properties [397]. In the drug industry, myrrh oleo gum resin may be used as directly compressible tablet excipient and release retardant [398] and as binder, release retardant and mucoadhesive for the preparation and evaluation of controlled release bioadhesive matrix tablets of domperidone [399].

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