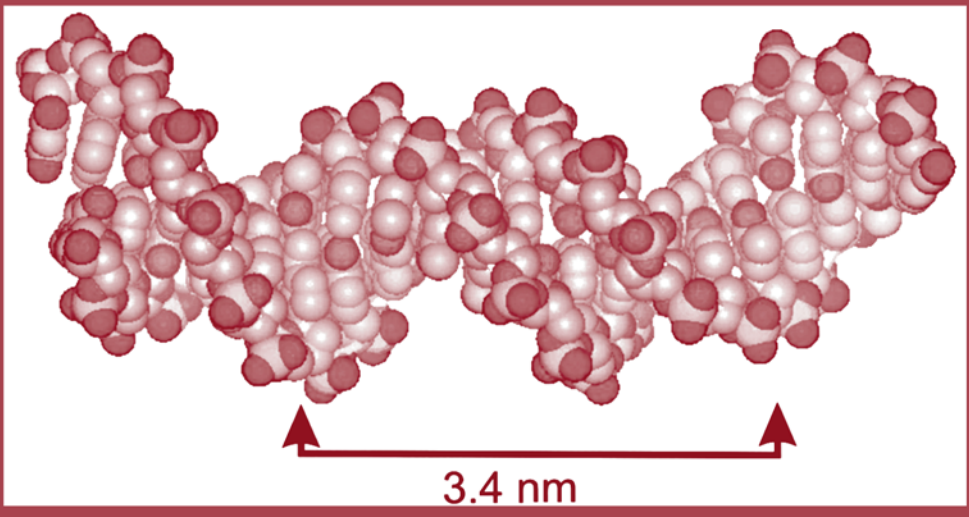
BIOMATERIALS *for* DELIVERY *and* TARGETING *of* PROTEINS *and* NUCLEIC ACIDS

EDITED BY

Ram I. Mahato



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Dedication

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

Preface

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

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

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

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

About the Editor

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

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

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

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1 Structure, Properties, and Characterization of Polymeric Biomaterials

Anjan Nan and Hamidreza Ghandehari

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1.1 INTRODUCTION

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

in Chapter 6) for acceptance in the biological system. Polymeric constructs are a major component of biomaterials.

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

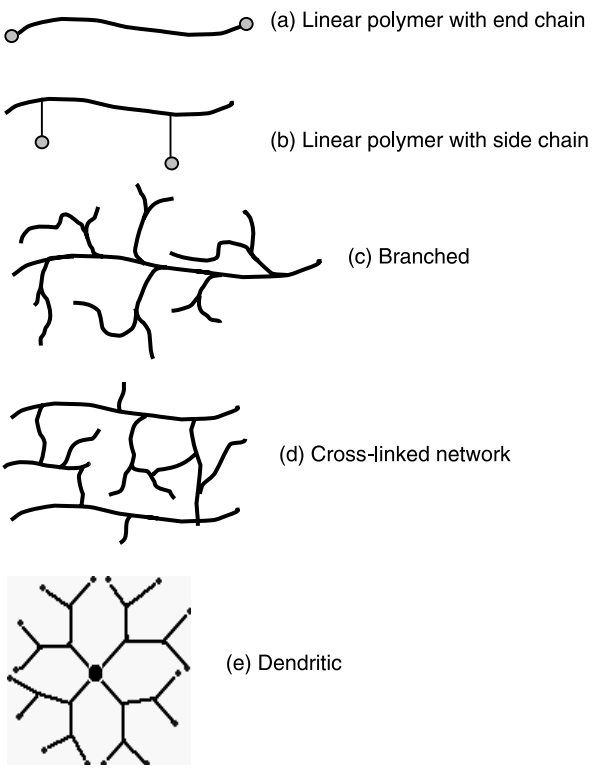
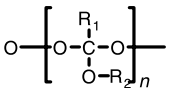


FIGURE 1.1 Various types of polymeric architectures: (a) linear with end-chain; (b) linear with side-chain; (c) branched; (d) cross-linked and (e) dendritic. Drugs can be attached to or dispersed in these polymers.

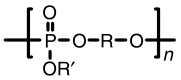
TABLE 1.1
General and specific structures of some commonly used polymers in drug delivery

Synthetic biodegradable polymers

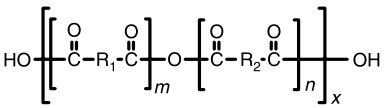
Poly(ortho ester)



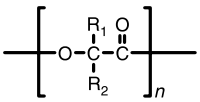
Polyphosphoesters



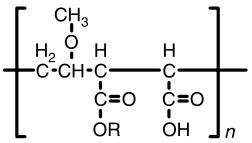
Polyanhydrides



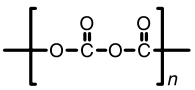
Polyesters



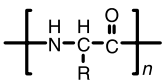
Methyl vinyl ether and maleic anhydride copolymer



Polycarbonates

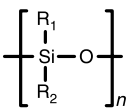


Poly(amino acid)s

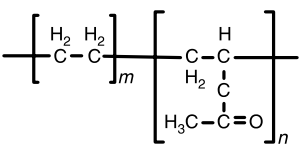


Synthetic non-degradable polymers

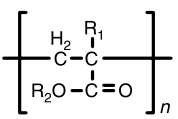
Silicone elastomers



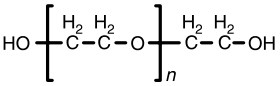
Poly[ethylene-co-(vinyl acetate)]



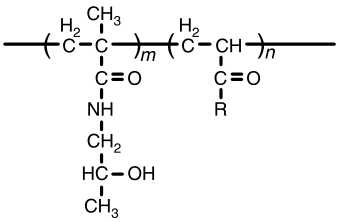
Acrylic polymers



Poly ethylene oxide/Poly ethylene glycol

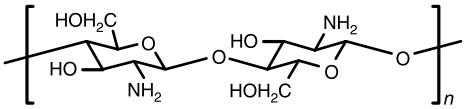


N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer

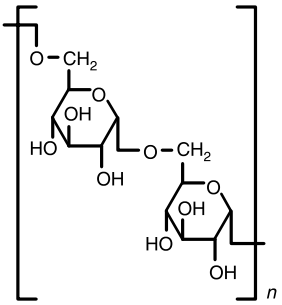


Natural and protein based polymers

i. Saccharides
Chitosan

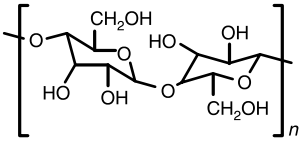


Dextran



ii. Collagen (typical amino acid sequence) $[(Gly)\text{-}X\text{–}Y]_n$ (X is often Proline, Y is often hydroxyproline)

iii. Cellulose derivatives



Genetically engineered polymers

Elastin-like polymers	$[VPGXG]_n$ (X = any amino acid except proline)
Typical silk-like polymers	$[(GAGAGS)_9$ GAAVTGRGDSPASAAGY$]_n$]
Silk elastin-like block copolymers	$[(GAGAGS)_m(GVGXP)_n]_o$ (X = any amino acid except proline)
[Poly(alanylglycine)]	$-(AG)_mX_n$ (X = any amino acid)

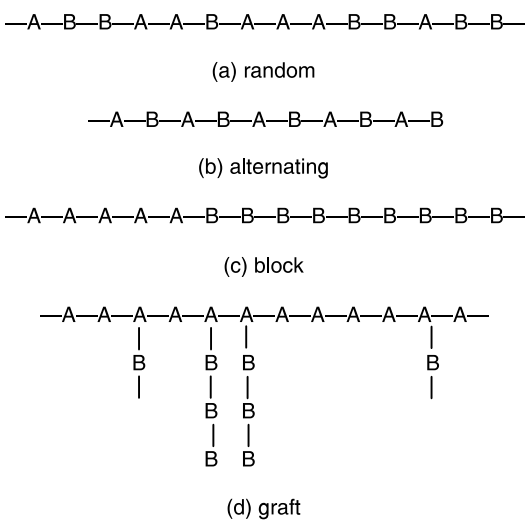


FIGURE 1.2 Various types of copolymers based on monomeric arrangements: (a) random; (b) alternating; (c) block and (d) graft. These structures comprise of A and B as two representative comonomers. Polymers can also be synthesized to contain more than two types of comonomers.

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

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

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

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

1.2 SYNTHETIC POLYMERS

1.2.1 Biodegradable Polymers

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

1.2.1.1 Poly(ortho ester)s

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

1.2.1.2 Poly(phosphoester)s

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

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

1.2.1.3 Polyanhydrides

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

1.2.1.4 Polyesters

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

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

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

1.2.1.5 Methyl Vinyl Ether-Maleic Anhydride Copolymer

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

1.2.1.6 Polycarbonates

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

of amino acids while maintaining mechanical strength similar to poly(BPA carbonates).

1.2.1.7 Poly(amino acid)s

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

1.2.2 Nondegradable Polymers

1.2.2.1 Silicone Elastomers

Silicone elastomers have been widely used for a variety of biomedical applications mainly as implants. These polymers have excellent mechanical properties besides being chemically stable. The stability of these polymers towards hydrolysis originates from their hydrophobicity. The polymers are available with a variety of molecular weights, degrees of crosslinking and chemical modification. Although polysiloxanes are mostly used in industrial applications such as lubricants, they are also widely used in biomedical and drug delivery applications. As implants they have received FDA approval for uses such as breast and heart valve prostheses. Several clinical products have been developed for controlled delivery of pharmaceuticals from silicone tubes such as SILASTIC® (Dow Corning, Midland, MI, USA) for controlled release of Levonorgestrel, a contraceptive hormone.[28] Norplant devices based on silicone elastomers have been used for providing controlled contraception over 5 years in women after forearm implant.[29] Silicone polymers have drawn much attention because of the popularity of breast implant devices. Studies in the past 10 years increasingly suggest substantiated risks of these implants such as local inflammatory and scarring reactions, and local infection. Since 1992 FDA has largely controlled the use of such biomedical polymers, although there does

not appear to be any evidence of systemic reaction, or abnormalities of the immune system in subjects who have received silicone implants.

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

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

1.2.2.3 Acrylic Polymers

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

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

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

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

1.2.2.5 *N*-(2-hydroxypropyl)methacrylamide

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

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

1.3 NATURAL AND PROTEIN-BASED POLYMERS

1.3.1 Polysaccharides

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

1.3.1.1 Chitin and Chitosan

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

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

1.3.1.2 Dextran

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

1.3.2 Albumin

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

1.3.3 Collagen

Collagen is the major structural protein found in animal tissues. Because of its unique structural properties it has found a variety of biomedical applications such as sutures, wound dressings, facial reconstructive surgery, and as drug delivery vehicles.[64] It is biocompatible and nontoxic to most tissues and has been used as films for ocular delivery of several ophthalmic drugs such as pilocarpine,[65] and antibiotics such as tobramycin[66] with improved bioavailability. Collagen has also been used for protein and peptide delivery. For example, water-soluble protein fractions isolated from bone matrix have been incorporated into collagen matrix and shown to induce bone and cartilage formation *in vivo*.[67] Osteoinductive devices, comprised of biodegradable

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

1.3.4 Cellulose Derivatives

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

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

1.4 GENETICALLY ENGINEERED AND HYBRID BIOMATERIALS

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

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

1.4.1 Elastin-like Polymers

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

1.4.2 Silk-like Polymers

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

1.4.3 Silk Elastin-like Block Copolymers

Silk elastin-like block copolymers (SELPs) have periodic incorporation of elastin-like blocks within silk domains. This approach increases the solubility of silk-like polymers by reducing their total crystallinity.[88] SELP solutions of appropriate composition and concentration are liquid at room temperature and can be injected through fine gauge hypodermic needles. *In vivo*, they form hydrogels that are no longer water soluble[92] leading to controlled release of bioactive agents that might be incorporated in the polymer solution. Release of solutes from these hydrogels is dependent upon the molecular weight, charge, and solubility of the solute, its diffusion through the polymeric matrix, composition of SELP forming hydrogel, and the conditions under which release takes place.[93–95] The SELPs have been reviewed for their biosynthesis, structures, physicochemical properties, and biological fate.[96] The SELP based hydrogels have been investigated as vehicles for controlled release of small molecular weight drugs and proteins[94] as well as plasmid DNA.[95,97]

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

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

1.4.4 Hybrid Polymers

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

1.5 CHARACTERIZATION OF POLYMERS AND POLYMERIC DRUG DELIVERY SYSTEMS

1.5.1 Thermal Analysis

1.5.1.1 Differential Scanning Calorimetry

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

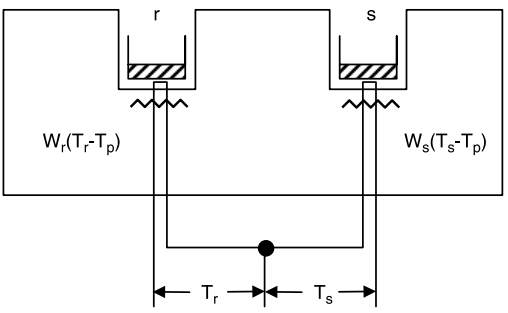


FIGURE 1.3 Schematic representation of a differential scanning calorimetry (DSC) apparatus: *r* and *s* are the reference and sample cells respectively; $W_r(T_r - T_p)$ and $W_s(T_s - T_p)$ are the power delivered to reference and sample cells respectively. T_r and T_s are the temperatures of the reference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

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

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

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

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

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

1.5.1.2 Thermogravimetric Analysis (TGA)

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

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

1.5.2 Determination of Molecular Weight and Molecular Weight Distribution

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

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

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

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

$$M_v = \left[\frac{\sum N_i M_i^{1+\alpha}}{\sum N_i M_i}\right]^{1/\alpha} \tag{1.5}$$

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

1.5.2.1 Osmometry

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

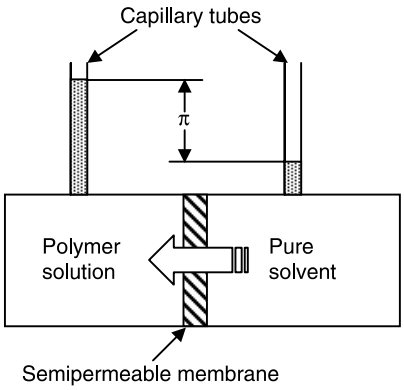


FIGURE 1.4 Schematic diagram of a membrane osmometer. In an enclosed system a polymer solution is separated from pure solvent by a semipermeable membrane that only allows passage of the solvent. The symbol π represents the osmotic pressure resulting from the concentration gradient.

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

$$\frac{\pi}{c} = \left(\frac{RT}{M_n} + A_2 c + A_3 c^2 + \cdots\right) \tag{1.6}$$

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

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

1.5.2.2 Dynamic Light Scattering

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

$$H\frac{c}{\tau} = K\frac{c}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2 c \tag{1.7}$$

where

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

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

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

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

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

$$\frac{K_c}{R_\theta} = \frac{1}{M_w} + 2A_2 c \tag{1.10}$$

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

1.5.2.3 Solution Viscometry

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

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

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

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

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

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

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

where k' is a constant.

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

1.5.2.4 Gel Permeation Chromatography

Gel permeation chromatography (GPC), also known as size-exclusion chromatography (SEC), is one of the most widely used methods for determining molecular mass average and polydispersity of polymers. In this case molecules are separated according to their hydrodynamic volume in solution when passed through a chromatographic bed of microporous gel particles. The gel material has a distribution of pore sizes ranging from 0.5 to 10^5 nm corresponding to the effective size range of polymer molecules. Separation of the molecules occurs by preferential diffusion of the different

sized molecules in a polymer mixture into the pores. Small molecules can permeate most of the pores and hence take a longer elution time. Larger molecules are mostly excluded by the pores and find their way out quicker through the interspaces between the gel particles. Selection of column packing material with the appropriate distribution of pore sizes is crucial for effective separation of molecules in SEC. Since the hydrodynamic volume of a polymer molecule in solution determines its ability to permeate a particular pore size, changing the solvent type or temperature can affect retention time in size-exclusion columns.

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

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

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

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

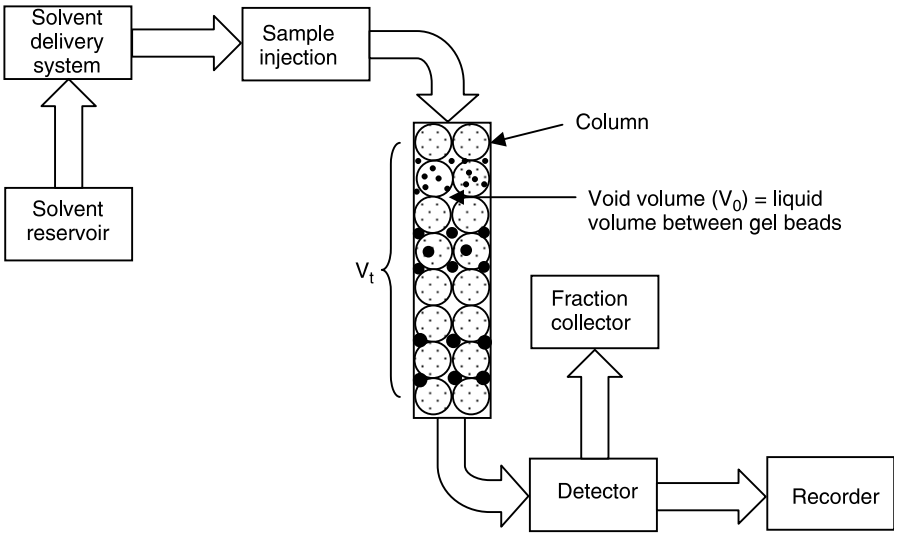


FIGURE 1.5 Schematic representation of a typical gel permeation chromatography instrument demonstrating the principle of separation by size-exclusion. Large molecules elute earlier than smaller molecules thereby producing a distribution of molecular weights for a given polymer. The molecular weight of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

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

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

1.5.3 Nuclear Magnetic Resonance

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

1.5.3.1 Determination of Stereochemical Configurations

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

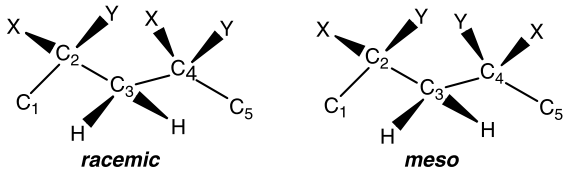


FIGURE 1.6 Stereochemical configurations of polymers. *X* and *Y* are different substituents, which can be arranged in different asymmetric arrangements.

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

1.5.3.2 Two-Dimensional NMR

2D NMR is a two-dimensional representation of the NMR spectrum. Compared to conventional one-dimensional NMR, 2D spectra have the advantage that the NMR parameters such as chemical shift and spin–spin coupling are observed separately. This means that overlapping spectra can be resolved, simplifying peak assignments. The 2D spectra may be presented in different forms; for example, J-resolved spectra or correlated spectroscopy (COSY). J-resolved spectra are represented by chemical shifts along one frequency axis and dipolar coupling constants along the other frequency axis. XCOSY presents the data as chemical shifts of different nuclei along the two frequency axes. The 2D NMR technique has been applied to a number of polymer systems such as propylene copolymers. In this method the 2D representation is the line-to-line correspondence between ^{1}H and ^{13}C splitting. The ^{1}H NMR spectra of polypropylene copolymers contain contributions from primary methyl ($–CH_3$), secondary methylene ($–CH_2–$), and tertiary methine (–CH) protons. Similarly, the ^{13}C spectra comprise contributions from the corresponding carbon atoms. The peaks in the 2D spectra are due to correlated absorptions involving simultaneous ^{1}H and ^{13}C splitting from the same group.

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

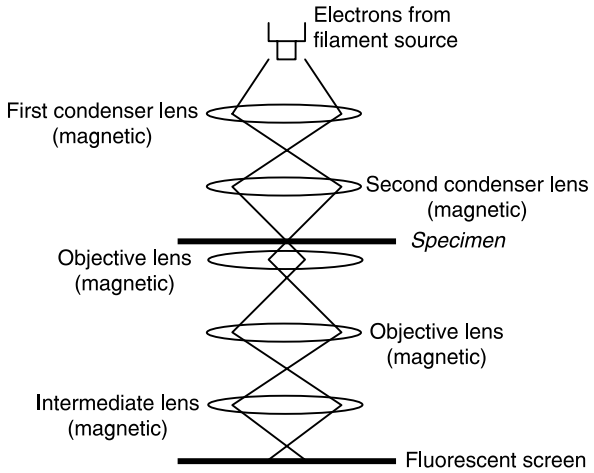


FIGURE 1.7 Simplified arrangement of optics in a transmission electron microscope (TEM). The electrons from a heated filament source are focused on the sample after passing through a series of condenser lenses. The beam after interacting with the sample penetrates and is focused on a fluorescent screen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

1.5.4 Microscopy

1.5.4.1 Transmission Electron Microscopy/Scanning Electron Microscopy

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

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

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

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

1.5.4.2 Atomic Force Microscopy

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

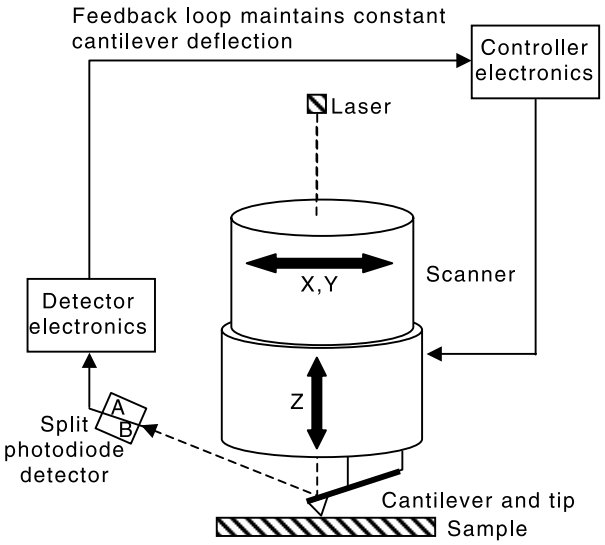


FIGURE 1.8 Schematic representation of an atomic force microscope. The microscopic probe consisting of a cantilever and a tip scans the sample (in X–Y plane). Alternatively the probe is fixed and the sample plane moves. The bending of the cantilever is obtained from the deflection (Z plane) of a laser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

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

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

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

1.5.5 Spectroscopy

1.5.5.1 Vibrational Spectroscopy

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

1.5.5.2 Infrared Spectroscopy

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

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

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

IR spectroscopy. The energies of molecular vibrations in IR analysis correspond to wavelengths in the range of 2.5 to 25 μm.

1.5.5.3 Raman Spectroscopy

Raman spectroscopy is concerned with detection of light scattered inelastically by molecules interacting with incident radiation. Interaction with the incident radiation of frequency ν_0 results in scattered light of frequency ν_0 (elastic/Rayleigh scattering) as well as $\nu_0 - \nu_{vib}$ (Stokes scatter) or $\nu_0 + \nu_{vib}$ (anti-Stokes scatter) when transition occurs from ground state to excited state or vice versa. The essential prerequisite for Raman spectroscopy is a change in the polarizability of the bond when vibration occurs.

1.5.5.4 Near Infrared Spectroscopy

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

The characterization of polymers by vibrational spectroscopy makes use of the concept that group frequencies, i.e., vibrational frequencies of particular chemical groups in molecules such as C=O and CH_3 tend to behave independently of the rest of the molecule. Thus the absorption frequencies of these groups appear in the same region of the spectra. By reference to standard correlation tables it is therefore possible in most cases to assign particular absorption bands to vibrations in the groups. In some cases it is possible to use internal calibration standards. For example, the vinyl acetate content of EVAc copolymers can be determined by measurement of relative intensities of acetate absorption at 1020 cm^{-1} and methylene absorption at 724 cm^{-1} and comparing with calibration curves for known compositions determined using other absolute methods. Kinetics of polymerization can also be estimated. For example, the reaction of isocyanates with amines or hydroxide groups to give urea or urethane can be easily followed by observation of the disappearance of the characteristic band of the isocyanate group at 1680 to 1610 cm^{-1}.

Vibration spectroscopy is usually performed using a variety of instruments such as dispersive IR spectrometers, Fourier transform (FT) spectrometers, and Raman spectrometers. In dispersive IR, the source, typically a black body (globar) source emits radiation over the whole IR region. Typical detectors include thermopiles, resistance thermometers (bolometers), Golay cells, or crystal diodes (measures in the microwave region). An attenuator is usually used in the path of the reference beam to equalize the sample and reference beam intensities. The movement of the attenuator is proportional to the sample absorption and displays as percentage transmission as a function of wavelength or wave number. FTIR spectrometers are used to investigate the far and near infrared region of the spectrum. Here the light source is a high intensity

mercury lamp, which emits a continuous spectrum. The system contains two mirrors (one fixed and one movable) and a beam splitter. The transmitted and reflected beams emerging from the beam splitter are incident normal to the two mirrors and following reflection is recombined at the beam splitter where they produce interference effects. The beam is then passed through the sample on to the detector. The movement of the mirror causes relative phase displacement resulting in an oscillatory pattern or interferogram, which is a representation of the spectral distribution of the absorption signal reaching the detector. The movement of the mirror determines the resolution of the spectra in the different IR frequency ranges. In Raman spectrometers, because of the very low intensities of the inelastic Raman scattering, a high intensity monochromatic laser light source is used. The laser light is focused very accurately on to the sample with typical spot sizes in microns. The scattered light is observed at 90° to the incident beam using high-resolution monochromator and photomultiplier tubes.

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

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

1.5.5.5 Mass Spectroscopy

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

desorption,[131] and secondary ion MS (SIMS)[132] have overcome such problems and enabled detection of compounds of molecular weight up to 10^4 Da. Of the different MS techniques in application in recent times, two approaches have gathered most interest, namely electrospray ionization (ES) and matrix-assisted laser desorption-time of flight (MALDI-TOF) mass spectrometry.

1.5.5.6 Electrospray Mass Spectrometry

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

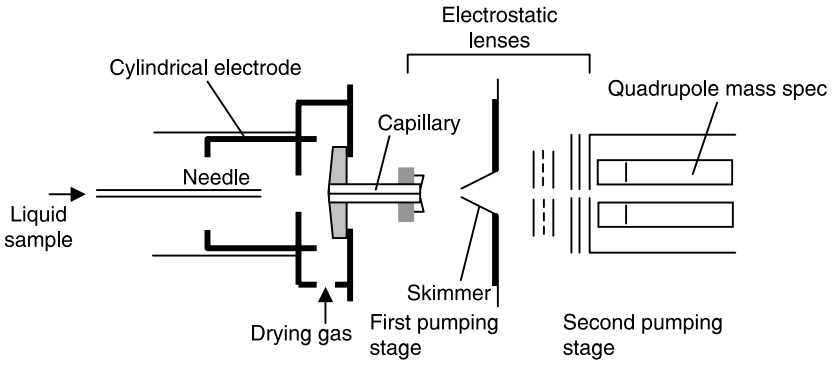


FIGURE 1.9 Schematic diagram of a typical electrospray mass spectrometry (ES/MS) system. The sample is injected through a needle housed in a cylindrical electrode kept at a high potential. The electric field generated at the needle point converts the sample to electrically charged droplets which evaporate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

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

The use of ES/MS for characterization of synthetic polymers is usually directed towards polar compounds, which are more easily ionized. Thus MS has been successfully applied for analyzing water-soluble PEGs up to 5 MDa and poly(amidoamine) (PAMAM) dendrimers of up to generation 10 (1 MDa).[133] However, apolar polymers like polystyrene[134] have also recently been successfully analyzed. In case of natural polymers (such as polysaccharides), usually ES/MS is performed after controlled degradation to oligomers. If suitably purified and by adjusting the potential of the skimmer,[135] it is possible to identify the different types of fragmentation and also analyze how single saccharide units are connected to each other.

The ES/MS is used to analyze the structure of peptides and proteins by determining the sequence of amino acid units, identifying protein metabolites, position of disulfide bridges, identifying covalently bound ligands and characterizing active enzyme sites.[136] These are achieved by peptide mapping/MS, which consists of analyzing the peptides obtained by protein fragmentation using MS and recording their spectra in a library.[136] ES/MS has also found application in nucleic acid sequencing[137] of various oligonucleotides, and identifying noncovalent associations in DNA–protein complexes.[138] MS interfaced with liquid chromatography (LC) (LCMS) is another widely used tool which enables analysis of macromolecules using a wide range of flow (from nanoliter to milliliter per minute).[139] Using ultrasonic nebulizers it is possible to achieve a suitable spray into the MS regardless of the solvent characteristics. Also LCMS can be interfaced with capillary electrophoresis (CE), which separates complex biological mixtures due to different charge/mass ratios of its components. The use of this method has found application in separation and identification of peptides and proteins including synthetic peptides,[140] glycoproteins,[141] oligonucleotides,[142] and oligosaccharides.[143] In the field of synthetic oligomers and polymer analysis, GPC is a powerful tool to determine molecular weight and polydispersity index. Mass spectrometry can be interfaced with GPC to analyze polymers.[144] In this way elution times of each species is associated with the corresponding molecular mass rather than the relative calibration standard curve that is commonly used in GPC.

1.5.5.7 MALDI-TOF Mass Spectrometry

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

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

In a MALDI-TOF spectrometer a pulsed laser such as Nd-YAG (355 and 266 nm), N_2 (337 nm) or TEA-CO_2 (10.6 μm) with pulse widths in the 1 to 100 ns range produces a simultaneous desorption in discrete packets of a large number of singly charged molecular ions (multiple charged ions are usually not observed), as proton or alkaline metal (Li, Na, or K) ion adducts. The ions are accelerated to a fixed kinetic energy by an electric potential applied just above the sample surface and the velocity of each ion will be proportional to its mass/charge ratio. By passing through a field free drift tube, ion species are separated into a series of ion packets each traveling with a velocity characteristic of its mass. A detector is present at the end of the flight tube which obtains the spectrum as a function of time.

MALDI-TOF is advantageous compared to ES/MS due to the absence of substantial fragmentation, high sensitivity (femtomolar), unlimited mass range (>1.5MDa), easy sample handling, and short analysis time. MALDI-TOF is used in a wide number of applications including structural characterization of polymers,[131,146] proteins,[147,148] oligosaccharides,[149] and oligonucleotides.[150] In determination of molecular weight distribution of polymers with large polydispersity, MALDI-TOF tends to underestimate the higher masses due to lower abundance, which overlaps with noise levels. This can be overcome by interfacing GPC with MALDI-TOF where the eluted fractions from GPC are analyzed offline by MALDI.[144,151]

1.5.5.8 X-ray Photoelectron Spectroscopy

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

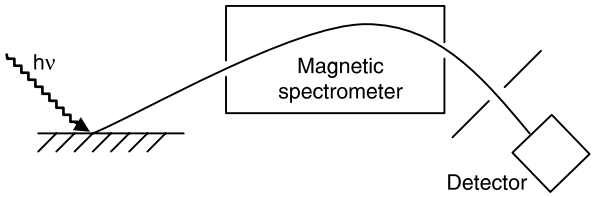


FIGURE 1.10 Schematic diagram of an x-ray photoelectron spectroscopy (XPS) instrument. A sample is bombarded with x-rays and the emitted electrons are passed through a magnetic field causing them to travel in a curved path to the detector. The binding energy of the ejected electrons is representative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

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

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

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

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

There are a large number of applications of XPS for polymer characterization in the literature. One mechanism that is studied extensively is oxidation effects.[152] Degradative phenomenon associated with oxidation, such as UV promoted photo-oxidation, primarily take place near the surface of polymers causing reduction of its mechanical properties. Normally the O 1*s* and the C 1*s* spectra are examined. For example the O 1*s* spectrum has a peak corresponding to the binding energy of about 533 eV with a shoulder at 535 eV. Upon desiccation in the presence of P_2O_5, this shoulder usually disappears and is thus attributed to absorbed water. The 533 eV peak is attributed to C=O and shows a small shoulder on the high binding energy side of the main C 1*s* peak in polypropylene. XPS has found application in distinguishing different carbon containing species on the surface of polymeric biomaterials.[153] XPS is also used

to characterize the extent of protein adsorption or binding on a variety of different biosensor interfaces which are modified with polymers in order to inhibit nonspecific adsorption from concentrated protein solutions.[154]

1.5.6 Gel Electrophoresis

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

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

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

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

1.6 CONCLUDING REMARKS

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

1.7 ABBREVIATIONS

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

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

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