BIODEGRADABLE SYSTEMS in TISSUE ENGINEERING and REGENERATIVE MEDICINE

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Preface

The development of materials for any replacement application should be based on the thorough understanding of the structure to be substituted. This is true in many fields, but particularly relevant in substitution and regeneration medicine. The demands on the material properties largely depend on the site of application and the function it has to restore. Ideally, a replacement material should mimic the living tissue from a mechanical, chemical, biological, and functional point of view. This agreement is, however, much easier to write down than to implement in clinical practice.

In the last few years, research in this field has been clearly evolving from the concept of substitution/replacement to the concept of regeneration. The use of prostheses, implants, and other nonliving materials to replace damaged or deteriorated tissues is expected to be replaced by the use of tissue-engineered viable constructs that will regenerate the injured tissues. This evolution, which is expected to lead to dramatic changes in health care in the upcoming years, will create an all-new range of therapeutic methodologies. That will only be possible due to the contributions of several different research fields, but it will not be possible at all without the use of a range of novel biodegradable polymeric systems. These systems will mainly be used in tissue engineering scaffolding and as carriers for the controlled release of a wide variety of bioactive agents (in many cases associating those two features in the same system). Nano-biotechnology, smart biomaterials, and stimulus-responsive materials, among others, will become increasingly more important as a result of the introduction of novel biodegradable polymeric systems into clinical applications.

Tissue engineering (TE) has emerged in the last decade of the 20th century as an alternative approach to circumvent the existent limitations in the current therapies for organ failure or replacement, which are mainly related to the difficulty of obtaining tissues or organs for transplantation. Conventional material technology has resulted in clear improvements in the field of regenerative/substitution medicine. However, despite the good results with the current methodologies, due to their severity, most of these injuries are still unrecoverable, creating a major health care problem worldwide. Consequently, there is a need for the development of advanced functional materials that are needed to improve the quality of life of thousands of patients suffering from tissue loss or tissue malfunctions. Only improved therapies may result in a decreased morbidity and mortality of patients with reduction of the overall costs of health care systems**.** This is particularly relevant if one takes into account the increasing aging of the population in the more developed countries, together with the increasing life expectancy, which has led to a tremendous growth of age-related problems.

Internationally, the combination of materials technology (namely using biodegradable polymers) and biotechnology is seen as the sector in which most major breakthroughs can be expected for medical devices in the coming future. Substantial gains are expected to be obtained both from a medical and economic standpoint as a result of this emerging technology. One of the main difficulties related to performing research in this area is the multidisciplinary approach of the teams. A strong group working on tissue engineering and regeneration must combine the expertise of materials scientists, polymer chemists, engineers, chemists, biologists, biochemists, etc. The problem is not only to join the correct team, but also to make team members understand all the requirements needed from the polymer and biotechnology side, thus generating synergies in their daily activities.

However, one should always keep in mind that the most important materials that are used in the development of adequate biomaterials for tissue engineering, replacement, and regeneration are based on polymers and their composites reinforced with bioactive ceramics. We decided to answer positively to the request from CRC Press to write a book titled *Biodegradable Systems in Tissue*

Engineering and Regenerative Medicine when we realized that there was a clear need for a textbook that would address in an integrated way many topics relevant to the field of the development and uses of biodegradable materials in biomedical applications, from the materials design to its processing, testing, and resulting applications. In fact, biodegradable polymer-based systems are playing and will continue to play a key role in tissue engineering, replacement, and regeneration in the near future. This increasingly more biologically driven materials science is believed to be one of the more appealing and well-funded research areas in the first decades of the 21st century. It will also create the need for the education of new scientists and engineers who are also "hybrids" and who can perform multidisciplinary research, combining materials and biotechnology.

One of the major features of the book is that it tries to address a range of relevant topics in an integrated and "looking forward" perspective. There are other books that cover similar topics, but they are in many cases collections of chapters by many different authors. This book presents the unique advantage of joining the view of only two of the most active groups in the field. They have collaborated for a long time and share many common visions, being very complementary (engineering + materials science + biology, and polymer chemistry + materials development/characterization). This, in our view, led to a book that is not just a collection of chapters, but instead a strong effort of integration. Therefore, we believe it will be a good research and education tool for final-year undergraduate and graduate students and faculty members around the world. The invited contributors were also carefully selected in order to add some complementary information to the book. They are well-known experts in their fields of research and were specifically chosen to cover a particular topic.

The book has the following main features:

- It provides an extensive and comprehensive description of biodegradable polymers used in medicine, their design, development, and processing. A wide range of biodegradable polymeric systems from both synthetic and natural origin is dealt with.
- Several chapters on the processing and applications of biodegradable systems, from injectable systems and injectable scaffolds to partially degradable polymeric and composite materials for orthopedics and dentistry, are included. The book also incorporates chapters on several methods for processing and testing biodegradable polymers and composites, including bioactive composites. Great attention is also given to the manufacturing of scaffolds, including overviews on solid free-forming techniques, fiber bonding, and methodologies based on particle aggregation, as well as on the processing of fibers, self-curing materials, and hydrogels that can be used to seed different types of cells. Finally, chapters on the characterization of the enzymatic degradation of natural-origin degradable systems and on the mechanical characterization of novel biomaterials are also included.
- A second less extensive section deals with the field of designing bonelike apatite coatings on biodegradable polymers by means of biomimetic coating technologies. The incorporation of proteins and other bioactive agents in such types of coatings is also addressed in one chapter.
- A detailed description of different applications of biodegradable polymers in the controlled release of bioactive agents is included. This includes a discussion on the possible release mechanisms that can be used and the ways to better use them in biomedical applications. However, the main focus is placed on the use of biodegradable controlled-release systems to deliver bioactive agents (such as regenerating factors) of use in the field of tissue engineering as well as on the chemical modification of such types of systems. Finally, a very detailed chapter on the uses of enzyme immobilization methods in biomedical applications is included.
- A discussion of the biological performance of biodegradable systems and the special requirements that should be taken into account when evaluating their biocompatibility is

also included. The cytotoxicity screening of materials and the problems related to testing biodegradable polymers are reviewed. Additional chapters deal with the very relevant topic of protein and cell interactions with degrading implantable systems, as well as with the mediation of the cytokine network upon implantation of such systems. Great attention is also given to the immune response to implanted natural-origin degradable systems and to several methodologies to tailor the cell adhesion and proliferation on the surface of biodegradable polymers, including the use of surface modification methodologies.

- The book concludes with a more applied part in which the reader is provided with a description on the use of biodegradable polymers in tissue engineering of a wide range of tissues: bone, articular cartilage, liver, skin, nerves, etc. It also includes a chapter on the design and use of biodegradable hydrogels in drug delivery and tissue engineering.

Finally, we must say that, as most of the readers would know, nobody can organize, write, and prepare this type of book for publication without the help of hard-working people and support from several institutions. We would like to acknowledge the contributions of the invited authors who accepted the invitation to write chapters for this book.

All the supporting institutions — namely the University of Minho and the Department of Polymer Engineering and CSIC, Institute for Science and Technology of Polymers — that have supported us, and our students, in so many ways also deserve a word of appreciation.

As main editor of this book, I am especially grateful to all of my postdoc fellows, Ph.D. students, and staff colleagues who work daily on the 3B's Research Group — Biomaterials, Biodegradables, and Biomimetics, which I (RLR) have the pleasure of directing. The outcome of this book is and will be mainly the result of their hard work, devotion, and commitment and of their own ambitions and aspirations. The book took more than a year to prepare, but is mainly the result of several main research lines that took many years to implement. All coauthors have put a great number of hours into this enterprise and realized that this was an important achievement for all of us. I cannot mention herein all their names, but you will see their names in the different chapters.

This book is dedicated to my wife Olga, my 3-year-old boy Bernardo, and to all my scientific kids (my students!) who made everything, including this book, possible. It is now time for us to move to other challenges. As Marie Curie once said, "One never notices what has been done: one can only see what remains to be done."

We hope the readers will enjoy the book as much as we enjoyed preparing it.

Rui L. Reis
Julio San Román

Editors

Rui L. Reis is 36 years old and was born in Porto, Portugal, where he still lives. At the present, he is an associate professor in the Department of Polymer Engineering at the University of Minho in the northern part of Portugal, where he is director of the 3B's Research Group — Biomaterials, Biodegradables, and Biomimetics. This is a research unit of *Excellence*, directly funded by the Portuguese Foundation for Science and Technology (FCT). Previously, he has been a lecturer at the Department of Metallurgical and Materials Science and Engineering, University of Porto. He was director of the undergraduate program in Materials Science and Engineering, and at the present, he is also director of graduate studies (M.Sc. and Ph.D.) in Materials Science and Engineering at the University of Minho. He was one of the primary individuals responsible for the creation and preparation of the new program in biomedical engineering that was started at the University of Minho in 2002. Furthermore, he is the Socrates/Erasmus (European student/staff mobility scheme) coordinator for Materials Science and Engineering at the University of Minho. He is also the director of R&D of the Cork Industries Holding of the AMORIM Group, one of the main economic groups with worldwide operations based in Portugal, where he directs a team fully devoted to the development of new cork-based products.

Dr. Rui L. Reis' education background includes (1) a degree in Metallurgical Engineering, University of Porto, (2) a master's degree by research in Materials Science and Engineering/Biomaterials, obtained in a joint program of the six major technical universities in Portugal, and (3) a Ph.D. in Polymer Engineering/Biomaterials, University of Minho, Portugal, a degree that was prepared in cooperation with Brunel University, London, United Kingdom. Rui L. Reis has been involved in biomaterials research since 1990. He has worked several periods abroad, in different universities and companies. His main area of research is the development of biomaterials from natural-origin polymers (starch, casein, soy, chitin, chitosan, algae, silk fibroin, etc.) that in most cases his group originally proposed for a range of biomedical applications, including bone replacement and fixation, drug delivery carriers, partially degradable bone cements, and tissue engineering scaffolding.

Lately the research of his group has been mainly focused on tissue engineering and drug delivery applications. He has been responsible for several cooperation programs, with universities and companies in the United Kingdom, the Netherlands, Spain, France, Finland, Germany, Italy, Turkey, Singapore, the United States, Canada, and Japan. Previously, he was responsible at the University of Minho for the European Union (EU) projects "ISOBONE — A Tissue Engineering Living Bone Equivalent," BRITE-EURAM III, which developed new tissue engineering strategies using starch-based scaffolds, and "ALGISORB — Algae Origin Bone Stimulators Enriched with Growth Factor," CRAFT, which developed new biomaterials fully from marine origin. At the present moment, he is the co-coordinator of a major EU research project, funded under FP6, the STREP "HIPPOCRATES — A Hybrid Approach for Bone and Cartilage Tissue Engineering Using Natural-Origin Scaffolds, Progenitor Cells, and Growth Factors," that has a 3M-Euro budget. Furthermore, he coordinates the only European Network of Excellence (NoE) on Tissue Engineering, "EXPERTISSUES — Novel Therapeutic Strategies for Tissue Engineering of Bone and Cartilage Using Second-Generation Biomimetic Scaffolds," which was approved on the first call of FP6. This highly funded NoE (budget of around 8M Euros) is composed of 20 partners, several being industrial, from 13 countries, and is expected to lead the way in all tissue engineering research in Europe. He is also responsible for several other projects funded by Portuguese, European, and American biomaterials

and polymeric industries and for a range of bilateral concerted actions. At present he is the principal investigator (PI) on grants totaling around 12M Euros.

As a result of these projects and other projects, he directed or is directing the work of around 45 (37 at the present moment) postgraduate researchers (postdocs, Ph.D. students, and M.Sc. students) from Portuguese, Spanish, Dutch, Slovak, Chinese, Bulgarian, Brazilian, Venezuelan, and Turkish origin, and he has been responsible for setting up two new labs at the University of Minho to carry out these activities. The researchers have a multidisciplinary background, including materials science and engineering, polymer engineering, chemical engineering, chemistry, biological engineering, textile engineering, biochemistry, biology and applied biology, medicine, and dentistry. In addition, four other members of staff at the University of Minho work in his research group.

As a result of his academic activities, Rui L. Reis has been awarded several prizes. The last two were the ESAFORM 2001 Scientific Prize for his work on the processing of starch-based biomaterials and the Jean LeRay Award 2002 by the European Society for Biomaterials for his outstanding contributions to the biomaterials field as a young scientist. In addition, he is a member of several editorial boards and acts as referee for a number of scientific journals, and has been presenting author, member of the scientific committees and organizing committees, referee, chairman, discussion leader in Gordon research conferences, and invited lecturer in many conferences worldwide (Japan, United States, Canada, Australia, Israel, Turkey, Cuba, Singapore, and a large number of European countries).

He was the director and the main person responsible for organizing the NATO Advanced Study Institute on Polymer-Based Systems in Tissue Engineering, Replacement, and Regeneration, held in Algarve, Portugal, October 2001. Furthermore, he was the chair and the main organizer of a special symposium on "New Challenges on Biodegradable Polymers," held in Tampa, Florida, during the annual meeting of the Society for Biomaterials (United States) in 2002. He was also the chairman of the workshop "Tissue Engineering: The Essential Elements," held in Reno, NV, in April 2003 during the Annual Meeting of the Society for Biomaterials (United States). He is also organizing a symposium for the World Biomaterials Congress to be held in May 2004 in Sydney, Australia. He was the director and main organizer of the NATO ASI on "Learning from Nature How to Design New Implantable Biomaterials: From Biomineralization Fundamentals to Biomimetic Materials and Processing Routes," held in Algarve, Portugal, in October 2003. He is editor of several international books and guest editor of several special issues of journals (*Journal of Materials Science: Materials in Medicine, Macromolecular Bioscience, Current Opinion on Solid State & Materials Science, Materials Science & Engineering Part C: Biomimetic and Supramolecular Systems*).

Dr. Reis has authored more than 100 papers in scientific journals, three books, five journal special issues, 80 book chapters in books of international circulation and in international encyclopedias, and more than 360 communications in conferences. He has also presented 35 invited lectures and is a member of 10 international research societies. His research work has been covered by news and interviews in the most prestigious Portuguese newspapers and radio stations and several times by national television.

Julio San Román studied chemistry at the University Complutense of Madrid (1972) and became a Doctor in Polymer Chemistry in 1975. He is the head of the Department of Macromolecular Chemistry at the Institute of Science and Technology of Polymers, CSIC of Spain and founded the Biomaterials Group at that institution in 1990. His scientific activities are centered in the study and development of polymer systems for biomedical applications, specifically tissue engineering, polymer drugs, and drug delivery systems. He has published more than 250 refereed articles in specialized journals — *Polymer Science, Biomaterials, Macromolecules, Biomacromolecules, Journal of Biomedical Materials Research, Journal of Biomaterials Science* — and related journals in the biomedical field.

He has contributed more than 20 chapters to specialized books and is co-editor of two books on biodegradable polymers for biomedical applications and biomaterials. In addition, he is the author of several patents on the application of bioactive polymers with antithrombogenic properties and self-curing bone and dental cements, some of which have been transferred to industry for development and commercialization. He has been invited to present lectures at more than 50 international meetings in the field of polymer materials, controlled release, biomedical polymers, and biomaterials. He has participated in several international projects, including EU-funded projects and the FP6 Network of Excellence on Tissue Engineering — EXPERTISSUES.

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Contents

PART IV Biocompatibility and Immunological Responses to Degradable Biomaterials

PART V Biodegradable Polymers for the Engineering and Regeneration of Different Tissues

Part I

Processing and Applications of Biodegradable Systems

1 Biodegradable Polymers in Medicine

Masakazu Suzuki and Yoshito Ikada

CONTENTS

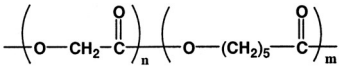
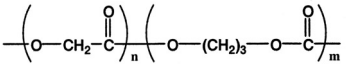
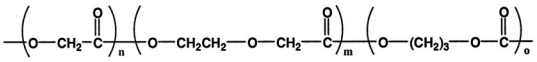
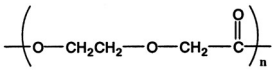
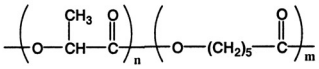
1.1 INTRODUCTION

Medical treatment not only for recovery of diseased regions, but also for restoration to normal functions is not an unrealistic ideal; it has become a practical target. Such medical treatment is called reconstructive surgery and regenerative medicine. Biodegradable polymers are used as materials for reconstructive surgery if the body itself has a potential for cure, because they are absorbed in the body after cure. If the body has no potential for cure, biodegradable polymers are inserted into disordered regions as a scaffold of cells for tissue regeneration, and they disappear after regeneration of tissues.

The first synthesized biodegradable product was poly(glycolic acid) (PGA), which has been studied and produced as a surgical suturing material. With the development of cellular engineering in the 1990s, there have been a number of studies on biodegradable polymers as scaffolds of cells for tissue regeneration.

Natural polymers, such as collagen, which is degraded in the body by enzymes, have been also studied and clinically used. In this chapter, we focus on synthetic biodegradable polymers.

TABLE 1.1
Characteristics of Biodegradable Monofilament Sutures

Suture	Chemical Structure and Generic Name	USP Size	Strength (g)	Diameter (mm)	Tm (°C)
Monocryl	 Poly(glycolide-co-ε-caprolactone)	3-0	4,665	0.295	200.8
Maxon	 Poly(glycolide-co-trimethylene carbonate)	3-0	4,602	0.297	206.2
Biosyn	 Poly(glycolide-co-dioxanone-co-trimethylene carbonate)	3-0	6,404	0.309	175.5
PDS II	 Poly-p-dioxanone	3-0	3,590	0.298	100.1
P(LA/CL)	 Poly(L-lactide-co-ε-caprolactone)	3-0	3,135	0.312	155.1
Ethilon[a]	Polyamide (Nylon 6 and 66)	2-0	4,160	0.275	220.5
Prolene[a]	Polypropylene	2-0	4,033	0.276	166.2

[a] Nonbiodegradable.

Source: From Tomihata, K. et al., *J. Biomed. Mater. Res. (Appl. Biomater.)*, 58, 511, 2001. With permission.

1.2 MATERIALS

There have been a number of studies on novel synthetic biodegradable polymers, but most products consist of single polymers and copolymers that have already been used clinically, and many of them are synthetic aliphatic polyesters. Table 1.1 shows the chemical structure of bioabsorbable polymers, which have been clinically used.[1]

The period required for absorption markedly differs depending on materials, from 2 to 3 months in PGA to 3 to 5 years in poly(lactic acid) (PLLA). Therefore, different materials are used according to the purpose. The period required for absorption varies even in a single material, depending on the product form such as fiber, film, and sponge; structural factors such as molecular weight and degree of crystallinity; additives and impurities such as residual monomers; and applied regions such as intraosseous, subcutaneous, and intramuscular regions.

1.3 APPLIED FIELDS

Table 1.2 shows the regions where biodegradable polymers have been clinically used or studied, together with their functions.

TABLE 1.2
Functions and Applied Regions of Biodegradable Polymers

No.	Function	Example of Medical Devices
1	Adhesion and fixation of tissues	Suture, bone fixation material, adhesive
2	Support and reinforcement of other medical devices	Suture reinforcement material
3	Temporary substitutes for tissues	Substitute material for endocranium
4	Shape maintenance and isolation	Stent, membrane for prevention of tissue adhesion
5	Securing space for tissue regeneration	Guided tissue regeneration, guided bone regeneration
6	Scaffold for tissue regeneration	Skin, cartilage, bone, blood vessel

Materials Nos. 1 to 4 are for reconstructive surgery and Nos. 5 and 6 are for tissue regeneration. Absorbable materials are also used as carriers in drug delivery systems (DDS), but we are not addressing DDS carriers here.

The products and studies shown in Table 1.2 are described in more detail in the following section.

1.3.1 Biodegradable Polymeric Materials for Adhesion and Fixation of Tissues

1.3.1.1 Sutures

Table 1.3 shows the commercially available absorbable sutures.[2] The materials are shown in Table 1.1. PGA suture was the first bioabsorbable medical material in the world that was clinically applied and became commercially available. The strength of the PGA suture is reduced to about 50% in 2 weeks, and it disappears in the body in about 3 months. Degradation of sutures without glycolic acid is slow.

Monofilament suture is considered morphologically ideal, and all sutures that have recently been developed and come into the market are monofilament sutures. Since softness of materials is necessary for the monofilament suture, flexible copolymers are used. Figure 1.1 shows the *in vivo* hydrolytic behavior of the polymeric fibers shown in Table 1.1 as expressed by the retention rate of the tensile strength. This indicates that the degradation rate ranges widely from the high rate of a glycolic acid-ε-caprolactone copolymer to the low rate of poly (lactic acid). The factors determining the degradation rate include not only the chemical structure of materials, but also the molecular weight, degree of crystallinity, monomer content, and physical form. Generally, the degradation rate of poly(lactic acid) is low, and that of PGA materials is high.

TABLE 1.3
Commercially Available Absorbable Sutures

Generic Name	Suture	Shape	Time to 50% Reduction of Tensile Strength
Poly (glycolic acid)	Dexon™, Medifit™	Multifilament	2 weeks
Poly(glycolide-co-*L*-lactide)	Vicryl™ Polysorb™	Multifilament	2 weeks
Poly-*p*-dioxanone	PDS II™	Monofilament	8 weeks
Poly(glycolide-co-trimethylene carbonate)	Maxon™	Monofilament	4 weeks
Poly(glycolide-co-ε-caprolactone)	Monocryl™	Monofilament	2 weeks
Poly(glycolide-co-dioxanone-co-trimethylene carbonate)	Biosyn™	Monofilament	2–3 weeks

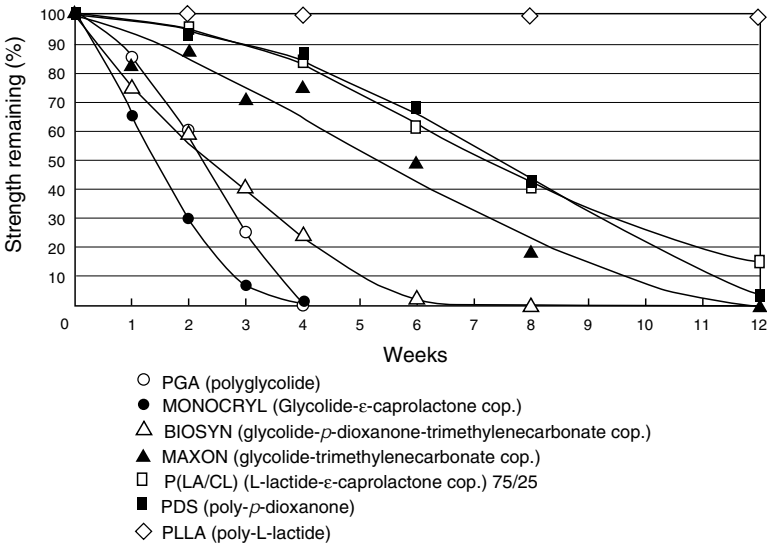


FIGURE 1.1 Tensile strength change of resorbable polymer in PBS at 37ºC with time.

1.3.1.2 Bone Fixation Materials

Metal fixation materials are used for internal fixation of fractured bones. However, since metal corrosion[3] sometimes occurred in the body, it was necessary to remove fixation materials after bone union. Furthermore, metal materials such as stainless steel cause artifacts in the commonly used diagnostic MRI, resulting in difficulty in the interpretation. On the other hand, ceramics with bioaffinity have problems in elasticity and fragility.[4]

To improve these drawbacks, bioabsorbable bone fixation materials have been developed. The materials used are poly(*p*-dioxanone) (PDS), PGA, PLLA, and their copolymers. Currently, bioabsorbable bone fixation materials are produced by more than 10 manufacturers in the world and are widely used clinically. PLLA is the most common material. High strength is required for bone fixation materials, and the development of a processing method for the production of bioabsorbable bone fixation materials with initial strength higher than the living cortical bone resulted in its rapid clinical application.[5,6] In Japan, bioabsorbable bone fixation materials are often used for screw fixation of transplanted bone in acetabular rotary osteotomy and replacement of artificial joints,[7,8] pin-fixation of osseocartilaginous fractures around joints,[9] and pin-fixation of dissociated ribs.[10] There are many types of bioabsorbable bone fixation materials, and these materials are used in surgical regions where resurgery would be required if metal materials were used for surgery, or in regions where resurgery is difficult to do. Recently, interference screws for the reconstruction of the anterior cruciate ligament and suture anchors for the fixation of the shoulder plate have been clinically used.

1.3.1.3 Adhesives

Table 1.4 shows the properties required for adhesives used in the body.

The most often used adhesive is fibrin glue made of blood-coagulating proteins, which are applied to many regions in various surgical fields. Since the most frequently used fibrin glue is produced from human blood, there is a risk of viral infections. Therefore, a number of studies on its substitutes have been performed. For example, GRF adhesive containing gelatin, resorcinol, formaldehyde, and glutaraldehyde; Advaseal™[11] consisting of copolymers of oligotrimethylene carbonate with polyethylene glycol and acrylate ester terminals, triethanolamine, and a photoinitiator esin Y; and Dermabond™ containing 2-octyl cyanoacrylate are used for skin adhesion.

TABLE 1.4
Properties Required for Surgical Adhesives

Available under moisture
Nontoxic
Pliable after cure, similar to soft tissues
Short in cure time

1.3.2 Biodegradable Polymer Materials for Support and Reinforcement of Other Medical Devices

To reinforce fragile tissues at the time of suturing, suture reinforcement materials are applied mainly for the lung and air tube in the respiratory apparatus and also for the hepatic parenchyma and digestive tract.[12] Small pieces called predgets were initially used for suture reinforcement and are at present being widely used in the automatic suturing device (mainly used in the U.S. and Japan) for fragile tissues such as the lung.

Fibrous cloth for tissues that can be applied in automatic suturing is increasingly used for fragile tissues. The typical biodegradable biomaterial used for this is Peristrips™ produced using bovine pericardium. The typical synthetic material is Neoveil™ made from PGA, which is used for sutures, while Peristrips™ is used for the pericardium. After biodegradable materials have achieved the desired reinforcement in sutured regions, they will be absorbed in the body, because it is unnecessary to maintain the initial strength.

Thoracotomy or thoracoscopic surgery is performed for the surgical treatment of the respiratory apparatus. The former is applied when a large area of tissues is excised and when it is difficult to obtain a sufficient visual field by thoracoscopy. In such cases, a large-sized fibrous cloth is required and fixed using fibrin glue. The techniques of thoracoscopic surgery are simple, and when the area of excision is limited, this method is effective and can be rapidly performed. The size of the materials used is not larger than that set to an automatic suturing device, and the load on the patient is small.

1.3.3 Biodegradable Polymeric Materials as Temporary Substitutes for Tissue

1.3.3.1 Substitutes for the Endocranium

When endocranial defects occur during craniotomy for the treatment of cranial nerves, they must be treated. Therefore, transplantation of autologous tissues, tissues collected from the same or different species, or synthetic dura produced using silicone and polyurethane has been performed. After the outbreak of bovine spongiform encephalopathy in 1996, Creutzfeldt-Jacob disease as a potential infection route of freeze-dried human dura drew attention, and its use was prohibited in Japan in 1997.

Currently, a white elastic e-PTFE sheet, Gore-Tex™, is the only artificial dura material used in Japan.

Artificial dura using synthetic biodegradable materials such as glycol acid–lactic acid copolymer (Vicryl mesh™[13]), and Vicryl mesh and collagen film complex[14] have been developed. These dura materials are advantageous compared to nonabsorbable materials when chronic inflammation reaction is absent, but they have such problems as too-early degradation and absorption before regeneration of connective tissues, and manipulation is more difficult due to their hardness. Currently, ETHISORB™ consisting of Vicryl mesh and poly-*p*-dioxone (PDS) film is commercially available in Europe. In Japan, an artificial dura, a complex of lactic acid-ε-caprolactone copolymer and PGA, was developed by a group at Kyoto University.[15,16]

1.3.4 Biodegradable Polymeric Materials for Shape Maintenance and Isolation

1.3.4.1 Stents

To improve the problems in plain old balloon angiopathy (POBA) using a balloon catheter, which was developed as a less invasive method for the treatment of coronary stenosis, the Wallstent was developed and first used clinically in 1986. A drawback of POBA is potential postoperative acute or subacute thrombosis. To solve this problem, metal stents have been developed. Thrombus generation can be suppressed by indwelling a metal stent while pressing the flap on the vascular wall. Postoperative subacute thrombotic obstruction was markedly improved by anticoagulant therapy with warfarin. Since the Palmatz–Schatz stent was proved more effective in preventing restenosis than POBA by random sampling tests[17] in 1994, many kinds of metal stents have been developed and are widely used all over the world. However, restenosis caused by intimal hypertrophy of smooth muscle cells occurring within 6 months after surgery could not be completely suppressed by metal stents, and this problem has remained unsolved despite the efforts made by many researchers. Recently, it has been reported that metal stents coated with suppressors of smooth muscle cell growth such as sirolimus are effective in preventing restenosis.[18,19] Since re-indwelling of stents is considered easy after reduction of the strength of stents by degradation, biodegradable stents have also been studied.[20] Tamai et al. performed clinical tests on PLLA coil stents and obtained good results.[21]

With the development of less invasive treatments, vessel stents have been clinically applied to not only the coronary artery, but also peripheral blood vessels, bile duct, esophagus, bronchi, ureters, and urethra. These stents, like stents for the coronary artery, are currently made of metal or nonabsorbable plastic. The production of stents using biodegradable materials has also been studied. To prevent temporary urethral stenosis during treatment for prostatomegaly, stents for the prostate developed by Petas et al. are used.[22]

1.3.4.2 Membrane for Prevention of Adhesion

Tissue wounds caused by injury or surgical treatment sometimes adhere to the surrounding healthy tissues in the process of healing. Adhesion of tissues often causes malfunction of the tissues or further injury. Various methods have been proposed to prevent adhesion. One method is to insert a biodegradable sheet so that it serves as a barrier separating the injured tissues and the surrounding tissues during the healing period and does not remain after that.

Adhesion-preventing materials must stay at the site in order to function as a barrier. If the material is fixed with sutures, the sutures cause adhesion. Therefore, it is important that adhesion-preventing materials adhere to the tissues themselves. Since they should not be hard materials to avoid physically stimulating the tissues, polymer materials with high water-absorbing capacity are used.

1.3.5 Biodegradable Materials for Securing Space for Tissue Regeneration

1.3.5.1 GTR, GBR

Periodontitis is one of the two major diseases in the dental field. The most favorable treatment is to recover the periodontium-supporting tissues (periodontal membrane) to the normal state. Gottlow et al. found in 1984 that the supporting tissues can regenerate if space for regeneration is provided.[23] The principle of the method differs from that of mandible reconstruction in that tissues can grow and regenerate simply by securing a sufficient space. Therefore, degradable and absorbable materials have been developed, such as GTR membrane using biological collagen,[24] and supplied as a single

material or in combination with other materials. Synthetic membrane materials available on the market include a lactic acid–glycolic acid copolymer,[25] a complex of this copolymer and collagen,[26] and a mixture of lactic acid and citrate ester.[27] These materials need longer periods (about 8 months) for degradation.

1.3.5.2 Artificial Skin

In 1980, Yannas and Burke developed an artificial skin material composed of a spongy sheet of collagen and chondroitin-6-sulfate, which is a kind of glycosaminoglycan (GAG), covered with a membrane of silicone.[28] An improved version of Stage I membrane is currently available on the market.[29] These artificial skin materials are also called artificial dermis to distinguish them from wound-covering materials. The artificial dermis is characterized by an inner layer of collagen sponge, which functions as a matrix to be replaced by regenerated tissues as its degradation proceeds. Therefore, its three-dimensional features such as pore size, as well as void volume fraction, degradability, and flexibility, are important in the process of wound healing.

1.3.6 Biodegradable Materials as a Base for Tissue Regeneration

There are two basic types of tissue regeneration in the regenerative medicine, the *in vivo* method and the *in vitro* method. Both methods require cells, growth factors, and a scaffold for tissue regeneration. In the former method, these materials are directly inserted into the diseased site, while in the latter method, they are inserted after formation of the tissues by tissue culture for a certain period. Absorbable materials are used as the scaffold in regenerative medicine because they are no longer needed after the tissues are regenerated.[30]

Table 1.5 shows the properties of biodegradable polymeric materials used as the scaffold for tissue regeneration.[31]

Different materials are used depending on the organ to be regenerated, but those studied as the scaffold are basically the same as the degradable and absorbable materials for tissue regeneration previously described. Researchers all over the world are trying to achieve better-designed materials with regard to cell adhesion (surface unevenness, charge, and hydrophilicity), substrate structure for cell growth (woven structure, knitted structure, nonwoven structure, and sponge), and maintenance of strength during the regeneration period and appropriate rate of degradation.

1.4 SUMMARY

Thirty years have passed since the PGA suture was first used in hospitals. Safety is the most important factor to be considered for medical materials directly applied to the human body, and this is particularly true of the degradable and absorbable biomaterials which are most commonly indwelled in the human body. Most of the degradable and absorbable biomaterials described in this chapter are designed as substitutes for the currently used materials in hopes of securing better quality of life (QOL). Since no other materials can substitute for degradable and absorbable biomaterials, their safety must be thoroughly examined. Because time is required for this, it will take some time for these materials to become clinically available on a large scale. We all hope to live a healthy life without excessive assistance. The degradable and absorbable biomaterials are being developed for this purpose, and they will soon become indispensable for reconstruction and as scaffolds in regenerative medicine.

TABLE 1.5
Physical Properties of Synthetic Biodegradable Polyesters Used as Scaffolds in Tissue Engineering

	Poly(glycolide)	Poly(*L*-lactide)	Poly(ε-caprolactone)	*L*-Lactide and Glycolide (10:90) Cop	*L*-Lactide and ε-Caprolactone (75:25) Cop	*L*-Lactide and ε-Caprolactone (50:50) Cop
T_m (°C)[a]	230	170	60	200	130–150	90–120
T_m (°C)[b]	36	56	–60	40	15–30	–17
Shape	Fiber	Fiber, sponge, film	Fiber, sponge, film	Fiber	Fiber, sponge, film	Fiber, sponge, film
Tensile Strength (MPa)	890 (fiber)	900 (fiber)	10–80 (fiber)	850 (fiber)	500 (fiber)	12 (film)
Young's Modulus (GPa)	8.4 (fiber)	8.5 (fiber)	0.3–0.4 (fiber)	8.6 (fiber)	4.8 (fiber)	0.9 (film)
ε_B (%)[c]	30 (fiber)	25 (fiber)	20–120 (fiber)	24 (fiber)	70 (fiber)	600 (fiber)
P_{wo}[d]	2–3 months	3–5 years	More than 5 years	10 weeks	1 year	6–8 months
P_{50}[e]	2–3 weeks	6–12 months	—	3 weeks	8–10 weeks	4–6 weeks

[a] Melting point.
[b] Glass transition temperature.
[c] Elongation at break.
[d] Period until the polymer mass becomes zero (in saline at 37°C).
[e] Period until tensile strength of polymers becomes 50% (in saline at 37°C).

Source: From Morita, S. and Ikada, Y., *Tissue Engineering and Biodegradable Equivalents*, Lewandrowski, K-U., Wise, D.L., Trantolo, D.J., Gresser, J.D., Yaszemski, M.J., and Altobelli, D.E., Eds., Marcel Dekker, New York, 2002, chap. 6. With permission.

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2 Injectable Biodegradable Systems

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CONTENTS

2.1 INTRODUCTION

Injectable systems are those systems that can be prepared immediately before being applied during a surgical intervention or, alternatively, that can be stored a long time without losing their key characteristic: injectability. In either case, they are defined as systems that, at room temperature or under ambient conditions, are able to be mixed, shaped, or handled and are subsequently implanted into the desired location inside the body (by injecting through syringe/needle or by using a spatula) and that harden, solidify, or jellify at body temperature/environment, becoming stable implants. The ease of preparation/application procedures and their versatility created a range of applications for injectables: tissue engineering of bone, cartilage, or other tissues; devices for the delivery of pharmaceutical components; bone cements; and bone defect filling devices.

Ideally, injectable formulations should preferentially involve physiological saline as the solvent (if any), should be based on macromolecular and hydrophilic precursors (hydrophilic macromolecules remain substantially outside the cell's plasma membrane), and should not liberate cytotoxic products during the transformation from liquid to solid and, later, during the course of degradation.[1]

This class of materials can be classified according to their stability (biodegradable, partially biodegradable, or inert systems) and shape (bulk implants or micro-/nanoparticles). This chapter will deal only with bulk implants based on biodegradable materials. Partially degradable devices and systems based on particles will be dealt with in other chapters of this book, while inert (nonbiodegradable) materials are beyond the scope of this book.

According to the method of preparation, handling, and *in situ* hardening, the solid biodegradable injectables can be further divided in several categories[2]: thermoplastic pastes, *in situ* crosslinking/polymerizing systems, *in situ* precipitating systems, and injectable gels, among others. The main properties, advantages, drawbacks, and examples of these systems will be discussed in the following sections.

It is not within the scope of this chapter to discuss the characteristics of spheres injection. It should be stressed, however, that the developments of bulk injectable, biodegradable systems were advanced because of some inherent disadvantages of the microparticles: the difficulties involved in removal of the particles after injection (if there are some complications during the treatment); the relatively complicated manufacturing procedures, involving in many cases the use of toxic solvents or heat and resulting in low yield; the need for reconstitution (suspension) of microparticles before the implantation; and the possible effects on the drug properties/efficacy caused by the solvents/heat used during incorporation. Moreover, even if these disadvantages are overcome and a suitable processing route is employed, the particles, after injection, can only perform one function: the release of the incorporated drug/factor/agent. However, in some instances, it is useful to have a device that not only can serve as a drug carrier, but also can fill tissue defects or serve as a scaffold for tissue regeneration. This range of function can only be performed by a bulk device, pointing to solid/semisolid injectables.

2.2 TYPES OF INJECTABLES

2.2.1 Thermoplastic Pastes

Thermoplastic pastes (TP) are polymeric systems with a low melting temperature (usually lower than 65ºC). These pastes are usually based in poly(ε-caprolactone) (PCL),[3] poly(lactides),[4] and poly(ethylene glycol) (PEG), alone or as copolymers or blends.[5] After melting, they are readily injected into the body, where they solidify in a few seconds after cooling to body temperature. These systems, due to their usually low molecular weight, are suitable for injectability even when heated slightly above their melting temperature.[2]

An adequate injectability behavior can be tailored by changing the relative amounts of the comonomers. For example, in block copolymers of poly(*D,L*-lactic acid)–poly(ethylene glycol)–poly(*D,L*-lactic acid) (PDLLA-PEG-PDLLA), while a PEG amount of 30% allowed for a gradual and continuous increase in the release of an anticancer drug, copolymers with 20% of PEG presented abrupt increases in the release behavior after some period of almost no release (Figure 2.1).[4] The stability of the solid after implantation may also be tailored by combining adequate amounts of slow- and fast-degrading monomers or by incorporating soluble additives, which can open channels and induce crack formation in the matrix.[3]

One of the main drawbacks of these systems is the high temperature needed to melt or soften the polymers. Even those polymers with low melt temperatures still can cause pain to the patient or generate some tissue necrosis, since their temperature, at the time of injection, is at least 20 to 25ºC above body temperature. An appropriate copolymerization/blending of different polymers can be the solution. Addition of 30% of methoxy-poly(ethylene glycol) (MePEG) in PCL brought down the melting point to 50ºC,[5] while 70% or more of lactide in PDLLA-PEG-PDLLA triblock copolymers avoided PEG crystallization, therefore vanishing with its melting endotherm and transforming the copolymer in a viscous liquid between 50 and 60ºC.[4] By optimizing the molecular weight of blocks of PCL-PEG-PCL triblock copolymers, it was possible to obtain formulations with melting

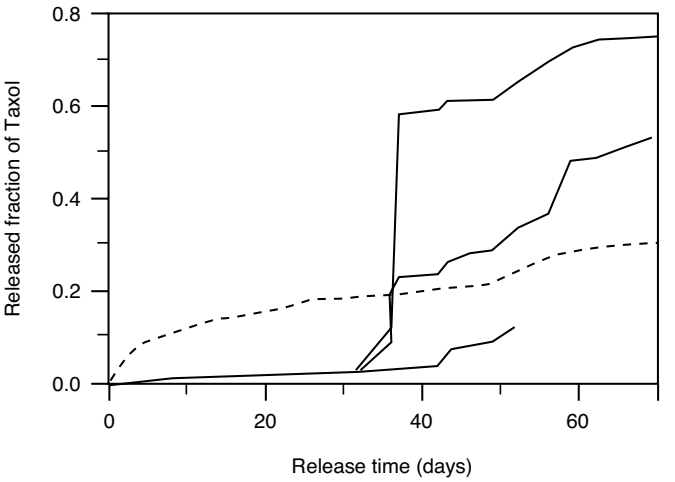

FIGURE 2.1 Cumulative release of taxol from 20%-taxol-loaded PDLLA-PEG-PDLLA cylinders into PBS albumin buffer at 37ºC. Solid lines: 20% PEG; dashed line: 30% PEG. (Adapted from Zhang, X. et al., *International Journal of Pharmaceutics*, 137, 199–208, 1996. With permission.)

temperature as low as 38ºC, which was considered suitable for injection into delicate sites such as the intraocular cavity.[6]

Some *in vivo* tests performed on such polymers showed that PCL, PDLLA-PLA blends, or PDLLA-PEG-PDLLA copolymers were able to release anticancer drugs at a rate adequate to reduce tumor growth in mice[3,4] and to produce an avascular zone in chick embryo chorioallantoic membrane.[3]

The release of an anticancer agent from PCL thermoplastic paste was higher in the presence of hydrophilic additives (such as gelatin or albumin); this increment was more accentuated if larger particles or concentrations of the additive were used. The release was found to occur through a coupled mechanism of diffusion through the PCL matrix, a transport through pores created in the matrix by the dissolution of the water-soluble additives or release due to erosion of the matrix. The particles would imbibe water, dissolve, or swell and exert a pressure that could rupture the polymeric matrix between adjacent particles, creating microchannels and thus facilitating the escape of drug by diffusion or convective flow.[3]

2.2.2 *In Situ* Crosslinking/Polymerization Systems

Typically, this class of injectables is formed by liquid monomers or macromers, a suitable polymerization/crosslinking initiator, and an activator source (chemicals, light, heat). This category encompasses a large range of materials. The most classical and known examples are the conventional acrylic bone cements, which have been studied for more than 40 years.[7] However, as they are nonbiodegradable, they will not be covered here. While chemical activation has been the preferred alternative for many years (following the original concept of bone cements), interest and research in photoinitiated systems is increasing.

These systems present several advantages: easy placement and subsequent polymerization to form devices with complex shapes that otherwise would be difficult to implant; improved adhesion of the polymer to the surrounding tissue, due to the injection of a liquid or viscous paste that will contact intimately with the micro-roughened surface of the tissue, generating a mechanical interlocking; and less-invasive clinical procedures, since the solution/paste can be injected with a needle and, in the case of photopolymerization, photocured with fiber optic cables.

Photopolymerization of degradable polymers was first presented around 10 years ago. One of the first works reported the development of a hydrogel barrier formed *in situ* for preventing

postoperative adhesion in animals.[8] Photopolymerizable systems present more advantages than the chemically activated ones. These include better control over the time of polymerization, which allows a reduction in the exothermic temperature of polymerization; spatial control of the polymerization sites, since the cure can be activated by fiber optic cables or transdermally; and choice of the injectable species, varying from liquids (to be polymerized inside the body) to viscous pastes (whose polymerization has previously started). As the light source can be chosen from a wide range available (UV, visible, laser), so can the polymerization time and the physical properties of the polymers. The network microstructure, formed during the polymerization and controlled by parameters such as macromer concentration, intensity of the light source, or exposure time, will strongly affect the degradation rate, swelling ratio, and mechanical strength.[9] Photopolymerized hydrogels are attractive for drug delivery applications due to their compatibility with hydrophilic drugs and with the human body and versatile control of the drug diffusion (by controlling swelling, degradation, and crosslinking density). As these hydrogels can be formed *in situ*, they will better adapt to the surrounding tissues. If loaded with adhesion-prevention agents, the drug delivery acts concomitant with the barrier effect provided by the hydrogel (see Section 2.4.2). The control of release can also be achieved by multilaminated photopolymerization, where each layer is polymerized with a different drug concentration, thus allowing one to tailor the system to disclose a specific behavior.[10]

Although these systems are very versatile and allow for a good control of the structure, being easily tailored for the desired application, several concerns should always be kept in mind: possible toxicity of monomers/macromers that will contact tissues *in vivo* either before the complete polymerization/crosslinking reaction or due to incomplete curing; possible damage to neighboring tissues caused by the temperature increase during the exothermic curing reaction; potential harm caused by solvents used as a vehicle (especially in the case of macromers); and the rate of polymerization, which should be fast enough for the material to harden in clinically acceptable periods of time. Nonetheless, different alternatives proposed in recent years were shown to be suitable for tissue engineering, drug delivery, and bone defect filling applications.

The acrylic bone cements, due to their well-established utilization and intense research carried out over 40 years, have served as the basis for the development of some of these systems. One proposed alternative relates to the partial substitution of a biodegradable component for poly(methylmethacrylate) (PMMA, the solid component of bone cements) while keeping the acrylic monomers as the polymerizable liquids. Both synthetic, such as PCL[11] and poly(propylene-fumarate) (PPF),[12] and natural-based polymers, such as starch-based blends,[13] were used to form these partially degradable systems (more information about these materials can be found in Chapter 4). Another approach regards the development of acrylic ester-terminated polymers from the conversion of polyol-terminated polymers.[2] Copolymers of *D,L*-lactide or *L*-lactide with ε-caprolactone were synthesized with acrylated end-groups and mixed with activators (*N,N*-dimethyl-*p*-toluidine [DMT] and benzoyl peroxide [BPO]); although they are liquid at room temperature, they hardened after 5 to 30 min inside the body.[14] Multifunctional monomers that can be photocrosslinked *in situ* to form degradable networks have been developed both for fracture fixation applications[15] and for cartilage tissue engineering and drug delivery systems.[16,17] The first group involves multifunctional anhydride monomers that react to form highly crosslinked and surface-eroding networks, while the second is based in high-molecular-weight macromers that produce loosely crosslinked and bulk-degrading hydrogels.

2.2.3 *In Situ* Precipitation

Another class of systems includes those designed to be capable of an *in situ* precipitation phenomena.

These injectable systems are solutions of polymers that, upon injection into the body, are precipitated due to contact with a nonsolvent (in this case, water from the physiological fluids). The hydrophobic polymer is dissolved in solvents considered physiologically compatible and that

are miscible with water (some examples include dimethyl sulfoxide [DMSO], *N*-methyl pyrrolidone [NMP], triacetin, etc.) and the solution is then easily injected into the body. When it contacts aqueous body fluids, the solvent diffuses out and the polymer, being water-insoluble, precipitates, forming a gel matrix and, later on, a solid polymeric implant. The vehicle serves not only as a solvent, but also as a plasticizer (decreasing the *Tg*) for the polymer. The most widely used polymers[2,18,19] are PCL and PLA (or copolymers of lactic and glycolic acid, PLGA).

The release of a water-soluble drug (as well as the consistency of the gel matrix formed) is dependent on the polymer concentration. Although the release occurs to water (body fluids), a study with PLGA implants showed that hydrophilic drugs may present slower release rates because they must partition from its hydrophilic environment (solvent) to a hydrophobic one (polymer) and then diffuse through the matrix into the aqueous medium.[19] Therefore, hydrophobic drugs are released faster; hydrophilic ones are released mostly from water-filled channels. Dispersed drugs are released faster than dissolved ones, since their dissolution creates a porous network of tortuous channels, giving rise to a dual mechanism of release.

Macromolecules (e.g., proteins) presented an opposite behavior, as they are too large to diffuse through the polymer. In this case, the release occurs only via diffusion through the interconnecting channels formed by the macromolecule in the matrix. Therefore, the addition of a hydrophobic material retarded the release of a hydrophilic protein, while the opposite effect was observed for water-soluble material (since these increased water uptake and, consequently, facilitated the movement of protein out of the matrix).[19]

The burst effect is also dependent on the polarity of the incorporated drug. For hydrophobic ones, the burst depends on the affinity of the drug for the solvent–water phase versus the solvent–polymer phase; on the other hand, for hydrophilic ones (injected as suspensions in the polymer-solvent solution), it depends on the number of drug particles that reside at the implant surface during polymer precipitation. As this is affected by the viscosity of the solution (which governs particle settling) and the degree of mixing of the solution, the burst effect turns out to be a complex and difficult-to-predict situation.

Although this is a very appealing system, due to the ease of injection and control of the properties, the reader should keep in mind some obvious problems:

1. Being a liquid in the first moments after injection, drug delivery systems will face a quick burst of the pharmaceutical component before the formation of the solid implant (this burst can be controlled by the concentration of polymer in the solution,[20] the molecular weight of polymer,[20] the choice of solvent, or the addition of a copolymer[21]).
2. The solvents used may raise concerns due to their possible toxicity and muscular damage when implanted subcutaneously or intramuscularly, since most toxicological data exists only for oral, parenteral, or intravenous administration of these solvents.[2]
3. The matrix will give rise to nonuniform implants with variable consistency and geometry and, as a direct consequence, the drug release from them will be variable and often unpredictable.[22]
4. If the drug is insoluble (or has small solubility) in the polymer/copolymer used, it must be dissolved in another solvent miscible with the polymer and mixed with the solvent for the polymer, increasing the complexity of the system and the chances of causing adverse reactions.[19]

For example, a copolymer of lactic and glycolic acid (PLGA) was formulated as an *in situ* precipitation system, by dissolving in glycofurol.[18] Several parameters (namely the copolymer composition, solution concentration, molecular weight, and protein loading level) were studied to determine their effect on the drug release rate. High amounts of protein (higher than 10%) generated numerous interconnected pores inside the matrix, greatly increasing the release kinetics, which for this system is controlled mainly by the erosion of the matrix. The typical burst effect on the release

was compensated for by an increase in the polymer concentration or molecular weight (both leading to a faster rate of coagulation/solidification of the polymer), leaving the long-term kinetics unaltered. No decrease in the biological activity of the protein (due to the polymer or its degradation products) was found.

These copolymers were also formulated for DNA delivery, allowing for an encapsulating efficiency approaching 100% and avoiding the usage of high-shear methods that could fragment the DNA.[23] These devices were well tolerated by the surrounding tissues, with only a modest foreign body reaction (similar to the ones observed for preshaped implants of the same polymers) around the implant. DNA release was faster from lower-molecular-weight polymers and occurred before complete erosion of the matrix, through a mechanism similar to protein delivery: burst due to the time lag for solidification, followed by diffusion through pathways connecting pockets of DNA to the surface and a later stage of release occurring subsequent to bioerosion of the polymer.

2.2.4 Injectable Hydrogels

The category of injectable hydrogels includes systems composed of aqueous solutions of polymers that, upon implantation into the body, form a gel and solidify. The most usual mechanisms of gelation encountered in these gels are the thermal one, where the gelation occurs due to an increase in the temperature from room temperature to body temperature, and the pH one, where the polymer jellifies when its acid (or basic) environment becomes a nearly neutral one (pH ~ 7.4) after injection.

In fact, pH-sensitive gels can be formulated with charged, water-soluble polymers, which jellify due to changes in the pH of the solution. As an example, chitosan solutions form a gel when the pH changes from slightly acidic to neutral. For instance, a pH-gelling cationic polysaccharide solution was transformed into thermally sensitive pH-dependent gel-forming aqueous solution, without any chemical modification or crosslinking, by using polyol-phosphate salts bearing a single anionic head.[24]

Usually, the thermal gelation happens due to changes in the balance of hydrophobic/hydrophilic interactions of the polymer with the solution or due to aggregations of polymeric micelles. The most well-known examples of this class of polymers are poly(*N*-isopropyl-acrylamide) (PNIPAAM) and poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymers. PNIPAAM is a nonbiodegradable polymer showing the phenomenon of lower critical solution temperature: a sharp and almost discontinuous transition from a sol to a gel at approximately 32ºC.[25]

Triblock copolymers of PEO-PPO-PEO have been extensively studied, and many different formulations are on the market under the trade name Pluronics® or Poloxamers®. However, they present several disadvantages. They are nonbiodegradable; their thermal gelation occurs only for concentrated solutions (typically higher than 16%), which can be toxic, especially when intended for intraperitoneal administration[2]; and they produce a significant increase of circulating cholesterol and triglyceride concentrations after injection in rats, resulting in hypertriglyceridemia.[26]

Another similar system is the block copolymer PEO-PLA, which jellifies when the temperature is brought down to 45ºC.[27] Although it is also water-based, it carries the inconvenience of high solution temperature (higher than 45ºC), which, for some specific kinds of drugs or for proteins, can be unaffordable.[27]

All the successful thermally sensitive sol-gel polymers present the advantage of being water-based, showing no problems of solvent toxicity. Moreover, most of them do not release any heat and just need to be warmed to body temperature to solidify. However, as there is a time lag between the injection and the gelling of the polymer (which will depend on the temperature of the transition) and as during this transition the shrinkage experienced by the polymer could release some amount of incorporated drug, a high initial burst could occur, drastically increasing the local concentration of the drug and decreasing the long-term efficacy of the system. Since the transition is driven by thermal conduction, it would be faster when the transition temperature is much lower than 37ºC.[28]

Therefore, when designing the gel, one should consider issues such as the gelation kinetics, the interface of the gel matrix with histogenesis, the matrix resorption rate, the possible toxicity of degradation products, and their elimination routes.[29] The gel kinetics, for example, is directly affected by the mechanism of gelation, the thermal one (limited by heat transfer) being faster than the pH or ionic ones (limited by mass transfer). This kinetics will have also an influence on cell spacing and distribution within the injected gel matrix. Drug delivery from hydrogels is affected by size, drug content, pore size, hydrophilic/hydrophobic balance, degradability, and the presence of specific interactions between hydrogels and the drug. In the case of biodegradable hydrogels, the release usually shows two stages: an initial one controlled by diffusion, and a later one controlled by both diffusion and degradation.

Another category of injectable hydrogels includes polymer solutions with a shear-thinning behavior. At rest, the polymer has a high viscosity, which decreases quickly upon shear, allowing it to be injected through a needle. After removal of the force, the viscosity increases again and a solid gel is formed *in situ*. The best example of such gels is hyaluronic acid. A clinical and histological study showed that stabilized, nonanimal hyaluronic acid was well tolerated and effective for soft tissue augmentation of the face, as an intradermal injectable implant.[30] The same polymer, when mixed with fibroblast growth factor 2 (FGF-2) and injected into bone defects in primates, promoted a significantly higher fracture healing (compared to untreated sites) as well as higher failure loads and energy to failure, demonstrating their suitability for bone repair.[31]

2.3 MATERIALS

2.3.1 PLA-Based Materials

PLA and PLGA (Figure 2.2) have been previously[22] used to deliver drug in a controlled manner as microspheres, microcapsules, films, implants, and nanoparticles. They have already been approved by the FDA for drug delivery and can be formulated as devices for other applications, such as orthopedic drug delivery.

PLGA copolymers rich in lactide are more hydrophobic; therefore, they absorb less water and degrade more slowly. Regarding the homopolymer, DL-PLA (amorphous) is preferred over L-PLA (crystalline) because it enables a more homogeneous dispersion of the drug in the polymer matrix. Both the homo- and copolymers erode through bulk degradation in aqueous environment through random hydrolithic cleavage of the ester linkages in the backbone.

Photopolymerization of poly(lactic acid)-*b*-poly(ethylene glycol)-*b*-poly(lactic acid) (PLA-*b*-PEG-*b*-PLA) or poly(lactic acid)-*g*-poly(vinyl acetate) (PLA-*g*-PVA) macromers with acrylate end-groups rendered a tridimensional network whose crosslinks were being homogeneously cleaved, systematically decreasing the crosslinking density of the overall network. The drug release behavior could be modulated: As the acrylate functionalization of the end-groups decreased, the initial crosslink density decreased and the degradation rate increased, both effects leading to an overall increase in the drug release.[32]

The PLA-*b*-PEG-*b*-PLA copolymers profit from the characteristics of each domain: PEG backbones give the material its hydrophilicity (which helps the dispersion of hydrophilic drugs, leading to more consistent release profiles), PLA blocks provide biodegradability, and the polymerizable end-groups provide the means to form a crosslinked network. Due to the high hydrophilic character of such hydrogels, the degradation kinetics of these PLA blocks is very different from that of linear PLA systems. In the linear ones, where the acidic degradation products catalyze further degradation, there is a first stage with no mass loss but with continuous decrease of molecular weight and a second stage where the mass loss occurs with a high rate that decreases until none of the original solid specimen remains. In the block systems, on the other hand, the mass loss rate is approximately linear during most of the degradation, only increasing during the last 20% of mass loss. This is due to the high degree of swelling of these systems, which lowers the concentration

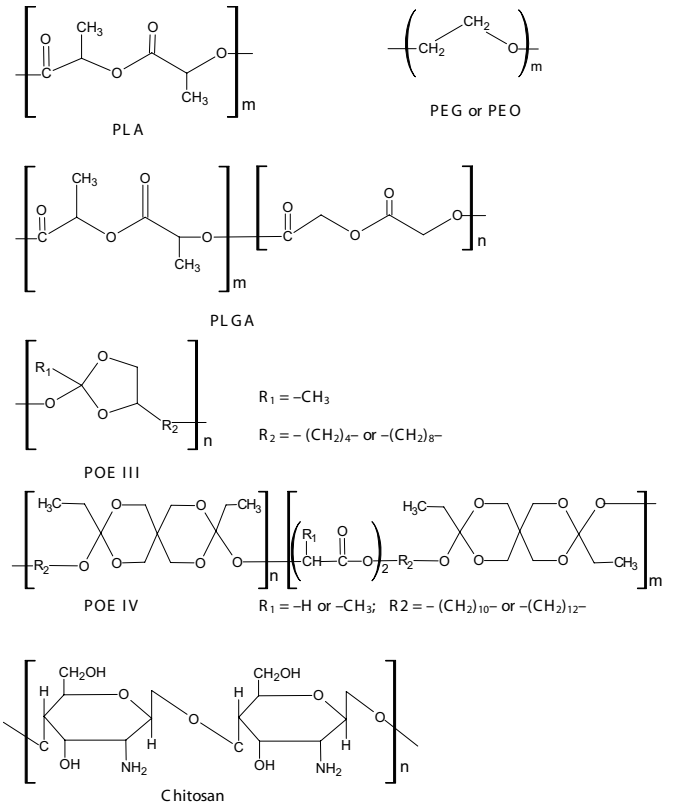

FIGURE 2.2 Chemical structures of some common polymers used as injectables.

of acidic species and allows more efficient removal of degradation products from the system, avoiding the acid catalytic effects.[9]

Copolymers of PLA and PEG were also employed as a thermoplastic paste. The release of a model drug (Taxol)[4] from PDLLA-PEG-PDLLA triblock copolymers could be via diffusion or degradation. The diffusion could happen by molecular diffusion in the polymer or through mass transport through open channels formed by connected drug particles (these channels are extensively formed when drug loading is higher than 30%[33]). The degradation mechanism is controlled by the relative ratios of hydrophilic and hydrophobic comonomers. With higher amount of PEG, the swelling was higher and the diffusion of the degradation products (acid oligomers) was approximately continuous, resulting in a sustained release of taxol without any abrupt change in the release profile. However, with lower content of PEG, the swelling was initially low and the diffusion of acid oligomers was low, creating a higher concentration of degradation products in the interior of the device (since in the outer region the diffusion path was shorter). As the acid molecules catalyze the degradation of PDLLA, a biphasic molecular weight distribution was observed, with a higher-molecular-weight shell and a low-molecular-weight core containing many oligomers and taxol molecules released during degradation. When the shell became too weak, it fractured and a high amount of taxol was released at once, creating a burst at some unpredictable and variable point during the course of degradation (see Figure 2.1).[4]

2.3.2 Poly(Ortho Esters)

Poly(ortho esters) (POE) are hydrophobic polymers that degrade by a surface erosion mechanism when immersed in water. Four generations were developed since 1970, but only the third and fourth

ones can be used as injectables. They possess ointment-like consistency, can be mixed with therapeutic agents or additives at room temperature, and do not need solvents or high temperature to be injected.[34] Since the ortho–ester linkage is acid-sensitive, the hydrolysis of the bonds on the surface (which decreases the pH) would catalyze further degradation. The third generation is weakly hydrophilic (Figure 2.2), being able to imbibe a certain amount of water; therefore, the degradation is relatively rapid.[35] The fourth generation (Figure 2.2) incorporates lactic acid units in the polymer backbone in order to control the degradation rate of ortho–ester linkages; initially the lactide units are hydrolyzed, catalyzing the hydrolysis of ortho ester, which proceeds until low-molecular-weight compounds are formed. There is a lag time for the starting of weight loss (controllable by the lactide concentration), and the degradation still follows a surface-erosion mechanism.[36] By varying the initial monomers, different degrees of hydrophobicity and viscosity can be obtained.

Due to the surface eroding behavior and hydrophobicity of these polymers, any incorporated substance (such as drugs) would be released concomitantly with this degradation and with a zero-order kinetics, that is, constantly and without any burst effect. The tailoring of the polymer molecular weight and of the physicochemical properties of the incorporated substances can be used to control the degradation: A higher molecular weight leads to a more sustained and longer release period.[37] Acidic drugs or substances were released faster (due to the acid-catalyzed acceleration of polymer degradation), while the basic ones stabilize the polymer backbone, allowing for a longer release. Similarly, hydrophobic drugs are released more slowly than hydrophilic ones, since the latter would help increase the hydrolysis rate and, consequently, the drug diffusion.[38] Additionally, for POE IV generation, the release (and erosion) rates can be varied by changing the relative amounts of the two diol units used during the synthesis and the nature of the R and R´ groups (Figure 2.1).[34]

To improve their biocompatibility, basic excipients were added to buffer the medium and neutralize the acidic degradation products and to prolong the polymer lifetime and drug release. The $Mg(OH)_2$ was the most effective additive, improving the biocompatibility of POE intended for ophthalmic applications.[39] This same study was also performed for PLGA injectable implants (which also degrade to acidic products), and the $Mg(OH)_2$ behave similarly. Additionally, it improved the stability of loaded proteins (smaller amount of bovine serum albumin [BSA] aggregates were formed) and their release (fourfold increase in the released amount of BSA after 30 days).[40]

2.3.3 Thermogelling Hydrogels of PEG and PLGA

Aqueous solutions of PEG-PLGA-PEG triblock copolymers are a free-flowing sol at room temperature, but become a transparent gel at body temperature. They are biodegradable and maintain their integrity for more than 1 month in rats, showing that the gelation rate is fast and that they have high enough mechanical properties. The higher the polymer concentration, the slower the release rate, due to tighter polymer–polymer contact among the gel. These triblock copolymers are thought to have a core-shell micelle structure in water, with PEG occupying the outer shell region to lower the free energy of hydration; the sol-gel transition occurs due to an increase in polymer–polymer attraction and micellar size, and the gel is formed by the packing of micelles.[41] As the polymer concentration increases, the transition temperature decreases due to an increase in the available physical crosslinking points that are dependent on polymer concentration. The transition can also be tailored by mixing two polymers (at the molecular level, i.e., forming mixed micelles) with different transition temperatures or by varying the ionic strength of the solution.[28]

More hydrophobic drugs will partition more into the PLGA micellar core and therefore will have a more sustained release profile; consequently, the longer the PLGA block (i.e., the higher its molecular weight), the slower the release rate, since the gel will absorb less water and degrade slower. For instance, in some works, hydrophilic drugs were released through a diffusion-controlled mechanism while hydrophobic ones were released through both diffusion and degradation mechanisms. Subcutaneously injected sol of PEG-PLGA-PEG triblock copolymers formed a gel in rats within a few seconds and provoked little or no tissue irritation at the injected site after 1 month.[42]

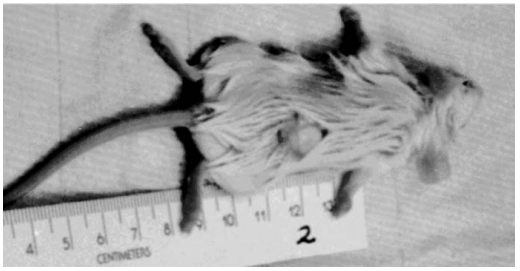

FIGURE 2.3 Round-shaped implant of a subcutaneously injected PLGA-*g*-PEG aqueous solution (29 wt%). (From Chung, Y.M. et al., *Biomacromolecules*, 3, 511–516, 2002. © 2002 American Chemical Society. With permission.)

The depot could stand long-term release of drugs, since its integrity persisted for longer than 3 months. There was 30% mass loss during this time, mainly from the PEG blocks. Due to this degradation, the gel became opacified and smaller (due to increased hydrophobicity and, consequently, lower water uptake). The PEG blocks allowed quick diffusion of the acid degradation products.[42] However, as the sol-gel transition temperature of these copolymers is strongly dependent on the PEG block length, the total molecular weight is limited to 4000 to 5000 g/mol if the transition is to appear at temperatures lower than 37ºC, allowing its use as a thermogelling system.[43] Therefore, new copolymers, PLGA-*g*-PEG, were developed that overcome these limitations. The sol-gel transition could be widely varied according to PEG content and length, and the duration of the gel could be controlled by mixing with a faster-degrading PEG-*g*-PLGA in different ratios.[43]

When adequately mixed, these two graft copolymers could allow for the tailoring of the durability of the gel from 1 week to 3 months[43,44] and do not show the limitations of the triblock copolymers in terms of molecular weight and degradation profile when the gel is designed to have the sol-gel transition temperature in the desired range (10 to 30ºC).[45] These graft copolymers were used in animal models as protein and cell delivery systems.[46] The release of insulin was effective in controlling the blood glucose level in periods ranging from 5 to 16 days, depending on the copolymer composition. The cartilage defect was notably repaired using chondrocyte suspension in PLGA-*g*-PEG compared with a control.[46]

PLGA-*g*-PEG was subcutaneously injected as a 29-wt% solution. The gel depot was round-shaped, indicating fast gelation kinetics, and persisted more than 2 months *in vivo* (Figure 2.3). The gel duration could be controlled by changing the structure: If PEG-*g*-PLGA was used, the absorption occurred within a month (probably due to relatively weak mechanical properties of the gel resulting from the flexible PEG backbone and the inherent small segments of PLGA diffusing easily out of the gel).[28]

2.3.4 Chitosan

Chitosan is an aminopolysaccharide obtained by alkaline deacetylation of chitin (cellulose-like polymer present in exoskeletons of arthropods). It is a biocompatible, biodegradable, mucoadhesive, pH-dependent cationic polymer (Figure 2.2). Neutralization of acidic chitosan solutions with glycerol-phosphate (GP) produced a thermally gelling solution with approximately neutral pH (6.8 to 7.2). The interactions responsible for this thermal sol-gel transition are[24,47] (1) increase of chitosan hydrogen bonding as a consequence of the reduction of electrostatic repulsion due to the basic action of the salt, (2) the chitosan–glycerol phosphate electrostatic attraction, and (3) the chitosan–chitosan hydrophobic interactions, enhanced by the structuring action of glycerol on water. The third interaction is probably the most important; at low temperatures, strong chitosan–water interactions protect the chains against aggregation but, upon heating, water molecules are removed by the glycerol moiety, allowing association of chitosan macromolecules. The reversibility of

gelation is dependent on the pH of the solution; when this is in the range 6.5 to 6.9, the gel is completely thermoreversible. The temperature of gelation increased as the deacetylation degree (DDA) decreased and is not affected by the molecular weight of chitosan.[24] The increase in gelation rate with higher DDA could be due to an increase in the crosslinking density between phosphate groups of GP and the ammonium groups of chitosan.[48]

Injectable gels containing chitosan (which is cationic) display tissue adhesiveness, since their surfaces usually bear net anionic characteristics. These gels were tested for cell delivery, being able to deliver active bone protein *in vivo* and leading to *de novo* cartilage and bone formation. Preparations containing several different cell lines were able to maintain more than 80% cell viability over extended periods of time *in vitro*.[24] Complexes prepared with low DDA chitosan were more stable at room/subambient temperature, but their gelation rate at 37ºC was decreased. Formulations with a reasonable gelation rate were not stable for more than 7 days at 4ºC or 24 h at room temperature. However, the gelation rate did not influence the release of a model compound (dextran). Another problem was the very fast and extensive weight loss (independent of gelation rate): Approximately 71% of the initial solid mass was lost in the first 4 h. Although the material could release drugs over a controlled period of several hours to a few days, sustained release over more than 1 week would probably require preincorporation of the drug into microparticles or liposomes for those molecules where release is only a function of passive diffusion.[48]

2.3.5 Other Materials

Alginate is an easy-gelling, biocompatible, and low-toxic material.[49] It can form hydrogels by ionic or covalent crosslinking. As those synthesized with ionic crosslinking present a limited range of mechanical properties and disintegration behavior, materials to be used as injectable tissue engineering matrices were developed with covalent crosslinking. Covalently crosslinked poly(aldehyde guluronate) (PAG), a polymer derived from alginate, presented a controllable degradation, dependent on the crosslinking density. The higher the crosslinking density, the lower the mechanical properties (due to increased number of network defects), the lower the degradation rate, and the better the bonding to bone.

Poly(ethylene glycol) (PEG) is a synthetic, hydrophilic, biocompatible polymer with intrinsic resistance to protein adsorption and cell adhesion. Peptides able to be cleaved by enzymes were used to design injectable, degradable hydrogels by copolymerization with PEG (as a BAB triblock copolymer terminated with acrylate groups). The acrylate groups could be photopolymerized, forming a hydrogel rapidly degradable in the presence of the targeted protease, but stable against other proteases.[50] They also incorporated cell adhesive peptides to achieve a biospecific cell adhesion. Smooth muscle cells seeded homogeneously in the hydrogels during the photopolymerization migrated through them and degraded the material due to the presence of the peptides, giving rise to a hydrogel with tailored biological activity by the appropriate combination of cell adhesive and proteolitically degradable peptides.[51] Cells were only able to adhere to and migrate through hydrogels containing both peptides; if either sequence was omitted, no migration was observed.

2.4 APPLICATIONS

The applications of injectable systems are many[1]: scaffolds for cell transplantation; barriers at the cellular or protein level to guide tissue regeneration; tissue adhesives or structural supports to bear mechanical loads during healing or regeneration; local drug delivery systems; provisional matrices (induction of cell migration to regenerate new tissue); and bone cements or bone filling materials.

Although the focus of the current chapter is the topic of injectables used for drug delivery systems, this section will present a brief description of other applications. Detailed information about other applications can be found elsewhere in this book (see, for example, Chapters 3 or 4).

Some injectables have already found commercial applications: PEG-based hydrogel precursors (*in situ* photocrosslinking) are already used clinically as surgical sealants.[52] Polyanhydrides are also already in the market as drug delivery systems in the brain after the removal of tumors.[53]

2.4.1 Ophthalmic Applications

Injectables are very attractive systems for delivery of pharmaceutical agents into the eye, since the current delivery devices suffer from drawbacks such as the need of a surgery for implantation (bulk preshaped devices) or the migration of injectable microparticles into the visual axis or into adjacent tissue sites.[6] Although these disadvantages are also present when the implantation occurs in other sites of the body, they are particularly dangerous in a delicate region such as the eye. Moreover, degradable injectables present advantages over conventional implants because repeated intraocular injections can increase the risk of infection, cataract, vitreous hemorrhage, or retinal detachment. The "peak and valley" effect of conventional administration could result in direct toxicity to ocular tissues followed by a rapid clearance of the drug.

2.4.2 Surgical Barriers

Photopolymerized hydrogels can be used as barriers to prevent adhesion of tissues after surgery. Barriers composed of degradable poly(ethyleneglycol-co-lactic acid) diacrylated macromers were highly resistant to protein adsorption and diffusion as well as to cell adhesion. Intravascular, interfacial photopolymerization of thin hydrogel layers was employed for prevention of thrombosis, while bulk photopolymerization on intraperitoneal surfaces was used for prevention of postoperative adhesion formation.[54]

2.4.3 Scaffolds for Tissue Engineering

For this application, injectable systems are particularly advantageous because they can fill any shape or defect (provided that they are formulated with the appropriate viscosity), can be easily formulated with cells by simple mixing, and do not require a surgical procedure to be implanted or, in the case of biodegradable ones, to be removed. Moreover, most kinds of injectables do not contain residual solvents (which may be present in preshaped scaffolds); those that do so usually employ physiologically acceptable solvents.

The mechanical properties of hydrogels can be tailored to match those of soft tissues, making them useful in the regeneration of soft tissues. Degradable hydrogels are advantageous because the cells are able to spread and migrate on or in scaffolds produced with them, while in inert ones, the cells are rounded and form clusters (Figure 2.4).[51] The cell proliferation and extracellular matrix production also reach higher levels in degradable hydrogels.

Since cartilage is composed mainly of water, devices intended for its substitution or for tissue engineering of cartilage should ideally possess high water content (desirable for transport of nutrients and waste) and be able to withstand the high loads that native cartilage experiences. Therefore, *in situ*-forming hydrogels are well suited for this application due to their aforementioned advantages, but also due to the high water content and tissue-like elastic properties of hydrogels. However, an obvious balance should be reached between cell viability and compatibility with native tissues (which require high water content) and maintenance of mechanical properties with the time (which require decreased water content and slower degradation rates). It was demonstrated[32] that both the PEG and PVA-based networks incorporating PLA were suitable for this application. Both growth factors and chondrocytes were encapsulated in the matrix, and the cells were metabolically active after 8 weeks.

Photopolymerized, crosslinked networks of degradable polymers such as PPF or polyanhydrides are suitable for bone tissue engineering because of their high mechanical properties, which match

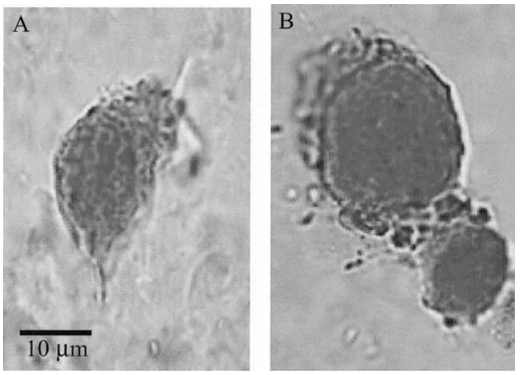

FIGURE 2.4 Human aortic smooth muscle cells growing in degradable (A) and inert (B) PEG photopolymerized hydrogels. (Reprinted from Mann, B.K. et al., *Biomaterials*, 22, 3045–3051, 2001. © 2001 Elsevier. With permission.)

(or surpass) those of trabecular bones. For an extended discussion on these materials, please see the next chapter.

2.4.4 Bone Cements

As previously mentioned, bone cements are the most widely used application of inert injectables and are the basis for the development of several degradable systems. Although fully degradable injectables have not yet achieved the requisites to be approved as bone cements, systems being proposed for bone supports, bone filling, or trabecular bone regeneration are being called bone cements.

One example is a multipart bioerodible cement system based on the conventional chemically initiated polymerization (employing vinyl monomer + initiator + activator), but with the incorporation of hydrolytically degradable polymers (PLGA and PPF), which degrade to acidic products and which form a crosslinked network (PPF) reinforcing the system.[55] The mechanical properties of the system matched (or surpassed) those of trabecular bone. One week after implantation in rat tibia, there was extensive bone formation, which almost entirely replaced the synthetic material. Neovascularization and osteoblastic activity were also seen. After 7 weeks, the site resembled a normal rat tibia metaphysis.[55]

A more detailed discussion of these systems can be found in Chapter 3 of this book, and bone cements composed of partially degradable injectables are presented in Chapter 4.

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3 Injectable Polymeric Scaffolds for Bone Tissue Engineering

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CONTENTS

3.1 INTRODUCTION

The ultimate goal of tissue engineering is to replace, repair, or enhance the biological function of damaged, absent, or dysfunctional elements of a tissue or an organ. This goal may be accomplished using different strategies that may use scaffolds, cells, and growth factors, alone or in combination, to develop engineered tissues that can function as living biological substitutes for malfunctioning or lost tissues.[1–3] Many different strategies can be used to develop these engineered tissues. The selection of the best strategy for developing cell-material constructs for the regeneration of a specific tissue defect is determined by several factors, such as the technical feasibility, required construct properties, and construct interaction with the host.[1] Therefore, the selection of the appropriate tissue engineering approach will also help define the most adequate scaffold design format and required properties.[1] Although three-dimensional porous structures have been recognized as the most appropriate geometry to sustain cell adhesion and proliferation, several specific applications in tissue engineering may take advantage of other design formats or combinations of existing ones,[4] such as drug delivery devices that can also function as scaffolds for cell attachment.

In fact, as the demand for new and more sophisticated scaffolds continuously increases, there has been a growing effort to design materials that may play a more active role in guiding tissue development. Instead of acting merely as a substrate for cell attachment, these matrices are designed to accomplish other functions through the combination of different format features and materials.[5] A good example of this is the use of injectable materials that can act simultaneously as scaffolds for cell growth and as drug delivery devices. This approach may also include, for example, the incorporation of microspheres (with encapsulated cells, growth factors, or other therapeutic agents)

within the polymeric matrix. While either injectable or preformed scaffolds could provide the basis for these multifunctional devices, injectable materials possess several advantages, which will be discussed in the following sections.

Several injectable systems, based on both thermal and photochemical crosslinking of several different natural and synthetic polymers, have been developed for a wide range of tissue engineering applications — for example, systems based on poly(ethylene glycol) (PEG),[6] alginate,[7] starch blends,[8–10] fibrin glue,[11] polyanhydride,[12] chitosan,[13] and poly(propylene fumarate).[14,15] Some of these examples will be described in this chapter.

3.2 ADVANTAGES OF INJECTABLE SYSTEMS FOR BONE TISSUE ENGINEERING APPLICATIONS

One of the main advantages of injectable systems as tissue engineering devices is their ability to allow for the use of minimally invasive surgical procedures for their implantation in the body. These procedures minimize the damaging effects of large muscle retraction, reduce the size of scars, and lessen postoperative pain, allowing patients to achieve rapid recovery in a cost-effective manner.[11] Injectable materials can also be easily placed in complex-shaped or relatively inaccessible defects[16,17] and then be reacted to form a polymer with the exact required dimensions, necessitating little or no additional shaping or modification of the device.[18] These systems may, at the same time, provide immediate mechanical support to the healing tissue. Furthermore, there is usually a good adhesion of the polymer to the surrounding tissue due to their intimate contact and due to mechanical interlocking that often results from surface roughness.[18]

As previously mentioned, these systems also offer the possibility of incorporation of particles of various sizes (microspheres, nanospheres, etc.), which might encapsulate cells, growth factors, or drugs and deliver them to the target location at a desired rate. Alternatively, injectable systems may deliver osteoinductive factors and recruit cells from the surrounding host tissues, avoiding the need to preload the device with cells of a different origin. Additionally, at the time of the crosslinking

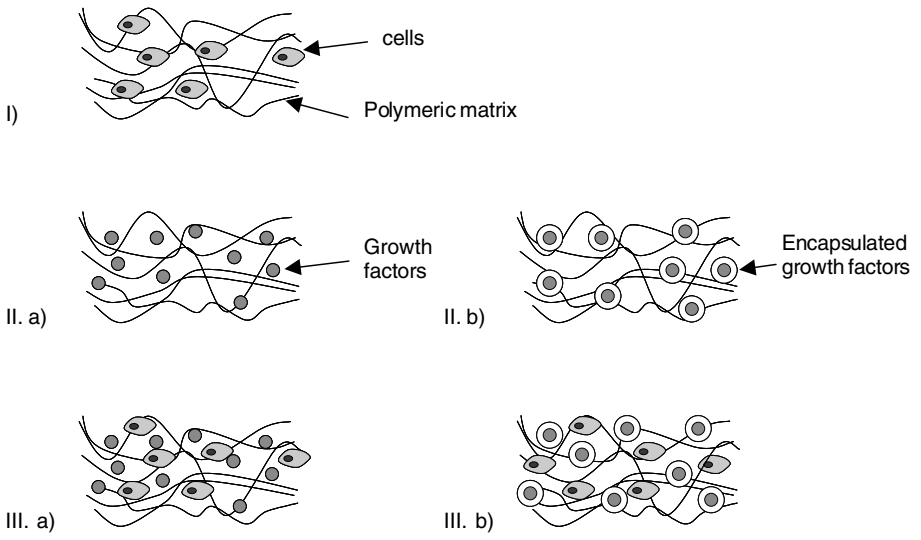

FIGURE 3.1 Bone tissue engineering strategies using injectable systems may be based on the delivery of cells (I), growth factors (II), or both (III). In these strategies, the growth factors may be directly incorporated into the polymeric carrier (IIa and IIIa) or previously encapsulated in microspheres (IIb and IIIb). The selection of the most appropriate material design depends mainly on the specific requirements of the target application.

reaction, it is also possible to incorporate leachable particles, such as NaCl, which create pores into which cells can proliferate and new tissue can grow, or a bioactive ceramic that can simultaneously provide enhanced mechanical properties and osteoconductive behavior.

Another approach for the use of injectable polymeric systems consists of the fabrication of scaffolds with complex three-dimensional architectures using stereolithography or other rapid prototyping techniques that make use of computed tomography or magnetic resonance image data to construct patient-specific devices. In this case, a negative may be fabricated from a dissolvable material (wax, for example), filled with the injectable polymer, and then polymerized. The negative is dissolved in an appropriate solvent, leaving a scaffold with the exact structure and dimensions required.[19]

Finally, crosslinked degradable scaffolds for tissue engineering offer a great versatility of properties, since the chemical nature of these networks can be modified through the manipulation of several parameters, such as the chemical structure of the precursor macromolecules and the crosslinking agents.[20]

3.3 CHALLENGES IN THE DEVELOPMENT OF INJECTABLE SCAFFOLDS

Biomaterials that are intended for use as tissue engineering templates must exhibit several general requirements, such as degradability, biocompatibility, high surface area/volume ratio, osteoconductivity, and mechanical integrity, in order to efficiently perform the function for which they are designed. As scaffolds for tissue engineering, injectable systems must meet additional requirements, related to the characteristics of the polymerization reaction, that are not often evaluated for other prefabricated biomaterials. These requirements regard the setting time and temperature change, the viscosity and ease of handling, and the toxicity of the monomers or solvent.[21] In fact, despite the advantageous features presented in the previous section, the use of *in situ* polymerizable systems poses some challenges, particularly regarding their *in vivo* application. For example, the range of physiologically acceptable temperatures is very narrow; however, most addition polymerization reactions are exothermic, generating large quantities of heat, which may result in some local tissue necrosis. Another concern arises from the use of toxic monomers or solvents, which may not react completely, leaving residues upon reaction in sufficient amounts to cause cell death.[18] The need for rapid processing and clinically suitable rates of polymerization in the water and oxygen-rich physiological environment constitute other restrictive aspects that must be considered when developing these systems.[18] Some of these limitations may be overcome using light photopolymerizable systems, which allow for better control of the polymerization processes. Consequently, photopolymerizable monomers or macromers have been generating interest, especially for applications involving cell transplantation.[20,22,23]

3.4 BONE TISSUE ENGINEERING STRATEGIES USING INJECTABLE SYSTEMS

3.4.1 Injectable Materials as Porous Scaffolds

Injectable porous scaffolds can be used in many situations in orthopedic surgery, when the priority is to restore immediately the mechanical stability. Injectable scaffolds may be created by incorporation of a porogen in the formulation, which is leached out after polymerization takes place, or by a reaction that releases a porogen gas, such as carbon dioxide. Poly(propylene fumarate) (PPF) is one such material that is currently under investigation for use as an injectable material in different bone tissue engineering strategies.[16,17,22,24–31] PPF is a linear polyester that can be crosslinked via free-radical propagation through its many unsaturated double bonds using a crosslinking agent,

such as *N*-vinyl pyrrolidone,[32] poly(ethylene glycol)-dimethacrylate,[24,28] or PPF-diacrylate,[28,31] and benzoyl peroxide as a free-radical initiator.

Several parameters of PPF-based systems can be tailored through manipulation of the method of synthesis, the crosslinking agent used, and the ratios of the initiator and crosslinking agent. Furthermore, at the time of the crosslinking reaction, two other components can be incorporated into the PPF: sodium chloride (NaCl), which creates pores into which new bone can grow, and tricalcium phosphate (TCP), which is an osteoconductive material that supports new bone growth.[16,24,25] The photocrosslinking of PPF using the photoinitiator *bis*(2,4,6-trimethylbenzoyl)phenylphosphine oxide (BAPO) and low levels of ultraviolet irradiation has also been investigated as the basis for the construction of an injectable bone tissue engineering scaffold that can be cured by light either during or after its injection.[22] Other studies have addressed the possibility of using commercially available stereolithography (SLA) processes to crosslink PPF with a suitable photoinitiator and thus generate scaffolds with a highly controlled external surface and internal geometry for bone tissue engineering.[26]

A different study[33] suggests a synthesis method of biodegradable macroporous hydrogels, based on poly(propylene fumarate-co-ethylene glycol) and prepared via coupled free-radical and pore formation reactions. In this case, the crosslinking was initiated by a pair of redox initiators, ammonium persulfate and *L*-ascorbic acid, and the porosity was obtained from the reaction between *L*-ascorbic acid and sodium bicarbonate, a basic component, which evolved carbon dioxide. This novel synthesis method allowed for the *in situ* crosslinking of biodegradable macroporous hydrogels with morphological properties suitable for consideration as an injectable tissue engineering scaffold.

3.4.2 Injectable Materials as Carriers for Osteoinductive Factors

Growth factors are proteins secreted by cells that act on the appropriate target cells to carry out a specific action.[34] They participate in a wide and complex network of cellular communications that affect essential functions, such as cell division, matrix synthesis, and cell differentiation. Several growth factors are expressed during different stages of bone fracture healing, and for this reason, it has been thought that they may serve as therapeutic agents to enhance bone repair.[34–37] These include members of the transforming growth factor-β (TGF-β) family, including bone morphogenic proteins (BMPs), as well as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF). Therefore, these proteins may find several potential applications in the repair of bone tissue, including the acceleration of fracture-healing mechanism, treatment of established nonunions, and enhancement of primary spinal fusion or treatment of established pseudarthrosis of the spine. The use of growth factors is also being explored for tissue engineering applications, especially for the reconstruction of critical size defects,[34] since a scaffold material alone may be inadequate to regenerate a large area of damaged tissue. However, in order to induce a specific biological effect, it is very important to select an appropriate carrier or delivery device system for a given growth factor.

To date, most of the work with tissue engineering using growth factor delivery has been based on the release of these molecules after their direct incorporation into 3-D porous scaffolds.[38] However, scaffolds with optimal porosity for cell proliferation and tissue ingrowth are sometimes unable to independently control the release of a bioactive agent through the porous network. Supplying the biological agents directly to the site of application is also rather ineffective, as the injected substance diffuses rapidly and therefore may not exert any long-term effects on bone induction or wound healing. For many tissue engineering applications, the sustained delivery of protein to a localized region of tissue is required because the distribution of proteins through the circulatory system can result in toxicity when delivered to nontarget tissues. In addition, many therapeutic proteins are large and penetrate slowly into bone tissue. Consequently, in order to maintain the desired therapeutic levels, it is necessary to use extremely high doses or frequent injections, which may not be desirable for an intended clinical use. To overcome these difficulties,

these bioactive agents may be loaded into microspheres, nanospheres, or hydrogels. Through incorporation into polymeric devices, a greater diffusion barrier is created, prolonging the length of time over which these molecules are released and thus act on the delivery site. However, these biological agents are also susceptible to denaturation, and therefore the processes used to fabricate these carriers and to load the biological substance are quite restrictive.

3.4.2.1 Injectable Materials Incorporating Nanospheres and Microspheres

Particulate polymers for the controlled delivery of macromolecules can be formed from many different degradable or nondegradable polymers. They can be produced in a wide range of sizes (1 to 100 nm for nanospheres and 1 to 100 μm for microspheres).[39]

Although they can be administrated by injection, microspheres alone are inherently limited as tissue engineering devices since they do not offer a substrate for cellular attachment and proliferation. Therefore, several studies[29,40] have addressed the possibility of incorporating nanospheres or microspheres into injectable polymeric matrices.

One of these approaches focuses on the incorporation of poly(*D, L*-lactic-co-glycolic acid)/poly(ethylene glycol) (PLGA/PEG) microparticles loaded with an osteogenic peptide (TP508) in a matrix composed of a mixture of PPF, PPF-DA (PPF-diacrylate), and sodium chloride (NaCl) particles.[29] This enabled the fabrication of an osteoinductive scaffold for bone tissue engineering capable of an appropriate release kinetics that can be adjusted to clinically desirable levels by varying the protein loading, PEG content, microparticle loading, and initial porogen fraction.[29]

Recently, researchers have investigated the *in vitro* release of transforming growth factor-β1 (TGF-β1) from gelatin microparticles incorporated in a novel oligo(poly[ethylene glycol] fumarate) (OPF) hydrogel.[40] This system can be crosslinked at physiological conditions within a clinically relevant time period.

3.4.2.2 Hydrogels

Hydrogels are potential candidates for many different biomedical applications, including tissue engineering, because of their water content, transport properties, and tissue-like physical and mechanical behavior.

Hydrogels for the release of biological agents can be divided into three types:[39] (1) physically entangled polymer systems that slowly dissolve and release the protein simultaneously with polymer dissolution, (2) chemically crosslinked polymer gels that degrade via hydrolysis or enzymatic digestion, and (3) hydrogels that swell after contact with water, allowing the diffusion of macromolecules throughout the entire matrix so that agents are released through a porous structure that expands during swelling. In the first two types of delivery systems, the release rate of the protein is mainly dictated by the degradation rate of the polymer, whereas in the last case, it is the size of the pores located within the network (which depends on the extent of crosslinking and degree of swelling) that will determine the protein release rate.

Several different hydrogels from both natural and synthetic origins have been developed, into which biological agents were successfully incorporated and then released to induce the desired therapeutic function.

Regarding bone engineering applications, BMPs were photoencapsulated in degradable PEG hydrogels.[41] *In vitro*, the release of this growth factor enhanced the expression of osteocalcin and type I collagen, and growth factors released from these hydrogels in subcutaneous tissue also induced the formation of ectopic mineralized tissue.[41]

Gelatin hydrogels have been studied for the release of FGF.[42] This study found enhanced bone regeneration in rabbit skull defects induced by hydrogels incorporating this growth factor, as compared to nonloaded hydrogels, where bone regeneration was ineffective. This effect was more evident in slower-degrading hydrogels, since these allowed for the long-term release of FGF.

3.4.3 Injectable Materials as Cell Carriers

Since bone healing involves migration, proliferation, and differentiation of osteocompetent cells, it is believed that the efficiency of an injectable system for orthopedic application could be significantly improved if it could be loaded with these cells. This approach can be very beneficial, especially for clinical situations in which the wound bed cannot provide these cells, such as in aged patients or in patients suffering from diabetes or severe trauma. In these cases, using the aforementioned approach, the bone formation will not be dependent on the local recruitment of osteocompetent cells. Furthermore, cell delivery can be useful in obtaining a long-term and economic solution to protein delivery by continuous production of endogenous proteins, such as growth factors and neuroactive chemicals, instead of the parenteral administration of these expensive drugs.

In order to successfully fulfill its mission, a cell delivery system must meet several design requirements:[43]

1. The cell carrier must preserve the viability and phenotype of the cell population during the crosslinking reaction.
2. The cell carrier must keep its mechanical integrity during injection and crosslinking of the system.
3. The cell carrier must allow for the attachment and functioning of the cells in the new environment, created after the complete crosslinking of the system.
4. The system should only keep cells encapsulated for short periods of time and then release them, allowing their interaction with the surrounding environment.

Nevertheless, mass transport properties, namely diffusion, in these systems are of primary importance in this approach, as the exchange of gases, nutrients, proteins, and waste products will directly affect cell survival within the matrix.

Cells can be temporarily encapsulated in hydrogels or non-hydrogel-based materials. Highly swollen hydrogels are capable of suspending cells in three dimensions, supporting nutrient diffusion to encapsulated cells. The employment of hydrogels as cell carrier devices also has the advantage of ensuring the delivery of an even distribution of a precise number of cells, as they are encapsulated within the system. Recent studies have explored the possibility of using a fibrin hydrogel as a delivery system for human mesenchymal stem cells (HMSCs).[11] Fibrin glue is formed from blood-derived products, namely thrombin, in the presence of calcium chloride and Factor XIIIa, and reacts with fibrinogen to produce a hydrogel. At optimized concentrations of fibrinogen and thrombin, the HMSCs loaded into this gel were able to spread and proliferate, and when implanted *in vivo*, the loaded cells were able to migrate out and invade a calcium carbonate-based ceramic scaffold, showing the potential of this material as a carrier device for HMSCs.[11] In a different study, human osteoblasts were isolated from calvarian bone explants and loaded into fibrin glue before seeding into biodegradable starch-based scaffolds.[44] The results obtained showed that cells were viable and that there were no signals of cell death after 3 weeks of culture. In addition, biochemical assays demonstrated that cells maintained the osteogenic phenotype throughout the experiment, and deposition of mineralized extracellular matrix could be detected.[44]

However, as previously suggested, during the crosslinking reaction of some systems, it is difficult to keep the conditions (namely temperature and pH) to a level that is not harmful for cells. For this reason, as for the delivery of biological agents, cell delivery systems can also be designed to incorporate microspheres that encapsulate cells, protecting them from adverse conditions during polymerization. In many cases, such an approach involves non-hydrogel-based scaffolding materials.

Recently, Payne et al.[15,45,46] tested the hypothesis that rat bone marrow stromal osteoblasts could be temporarily encapsulated in gelatin microspheres, protecting them from short-term environmental effects such as those associated with the crosslinking of an injectable polymeric carrier for bone tissue engineering. In this study, the cells encapsulated in the gelatin microspheres were incorporated

into PPF composites using N-VP as a crosslinking agent with crosslinking times of up to 10 minutes. Encapsulated cells showed higher viability than nonencapsulated cells, and the gelatin microspheres did not affect the crosslinking reaction, demonstrating the potential of such an approach for bone tissue engineering.[46]

3.4.3.1 Biomimetic Hydrogels

In spite of their ability to suspend cells in a 3-D manner and support nutrient diffusion to ensure cell viability, some hydrogels may not provide an ideal environment for anchorage-dependent cells such as osteoblasts. The attachment of various peptides to the surface of biomaterials has been found to enhance the adhesion of osteoblasts and simultaneously provide a biomimetic environment for cells encapsulated within hydrogels. The most widely studied adhesive peptide, Arg-Gly-Asp (RGD), is present in the cell-binding domain of extracellular matrix proteins. The integrins on the surface of the cells bind to RGD, enabling cell adhesion on surfaces, which would not provide adhesion otherwise. RGD groups have been incorporated into alginate gels,[7] *N*-isopropylacrylamide and acrylic acid hydrogels,[47] and poly(ethylene glycol) (PEG) hydrogels,[48–50] among others.

A water-soluble block copolymer poly(propylene fumarate-co-ethylene glycol) (P[PF-co-EG]) was recently developed, which has the potential to be crosslinked *in situ* into thin films[14] or macroporous three-dimensional scaffolds.[33] The crosslinked monomer has been modified in bulk with a covalently linked RGDS model peptide for enhancing the migration of marrow-derived osteoblasts.[51] A novel macromer, oligo(poly[ethylene glycol]fumarate) (OPF), has also been developed for tissue engineering applications. This macromer consists of two repeating units, PEG and fumaric acid, which can also be crosslinked in the presence of water to form hydrogels also incorporating adhesion-specific peptides spaced from the polymer network via a PEG chain.[52] The attachment of marrow stromal cells showed an increase with increasing initial peptide concentration and was mediated solely by receptor–ligand interactions as evidenced by competitive binding experiments with soluble peptides. The *in vivo* bone and soft tissue behavior of these novel OPF hydrogels was also assessed using a rabbit model.[53] The histological evaluation of implants proved that the OPF hydrogel is a promising material for use as an injectable biodegradable scaffold in bone tissue engineering.

Poly(ethylene glycol) hydrogels modified with adhesive RGD peptide sequences have also been investigated as encapsulation matrices for osteoblasts.[48] Modified surfaces of these hydrogels have been shown to dramatically increase the cell attachment.

These studies demonstrate that the interaction of hydrogels with incorporated cells can be designed to enhance *in vivo* bone formation through the modification with adhesive peptides, while nonmodified hydrogels have shown limitations as cell transplantation devices.[54]

3.5 CONCLUSION

In bone regeneration, as in the regeneration of any other tissue, a variety of growth factors act on cells, forming a complex system in which timing, site, and concentration of actions are carefully regulated by the whole organism.

Tissue engineering strategies require the design of materials that reflect the understanding of these biological interactions as much as possible, allowing the development of increasingly intelligent devices that can combine different functions. Injectable systems offer a great potential for applications in interactive bone tissue engineering approaches, as they can be designed with a wide range of properties and configurations and can incorporate cells and a number of different biological agents capable of different combined functions. This versatility also allows for tailoring of these materials to specific applications in bone engineering. The requirements for the final application of the materials will dictate the type of tissue engineering approach to use and consequently the type of construct needed.

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4 Totally or Partially Biodegradable Self-Polymerizing Composites for Orthopedic Surgery and Dental Applications

Blanca Vázquez, Gustavo A. Abraham, Carlos Elvira, and Julio San Román

CONTENTS

4.1 INTRODUCTION

Self-polymerizing resins are obtained at room temperature by the free-radical polymerization reaction of an acrylic monomer in the presence of a prepolymerized component. The process is initiated by the decomposition of a small amount of organic peroxides (1–3%), usually benzoyl peroxide (BPO), activated by the redox reaction with tertiary amines. Aromatic amines are the most effective compounds to generate the primary radicals and *N,N*'-dimethyl-4-toluidine (DMT) is the most frequently used.

Self-curing acrylic resins based on blends of poly(methyl methacrylate) (PMMA) particles and methyl methacrylate monomer, or copolymers of methyl methacrylate with styrene or other acrylic monomers, including ethyl or butyl methacrylate, are used in dentistry and orthopedic surgery for the fixation of endoprosthesis (i.e., total hip replacements and knee joints). This system has also been the primary constituent of the vast majority of artificial dentures for at least 50 years and has been used in medicine by neurosurgeons for replacements of skull defects. The blend of polymer/copolymer and monomer (or a mixture of acrylic monomers) forms slurry of relatively high viscosity, which can be manipulated easily or injected into the intraosseous cavity. The blend

hardens in the physiological medium by the free-radical polymerization of the monomeric components at physiological temperature.

Self-polymerizing biodegradable bone cements present many advantages over poly(methyl methacrylate) bone cements. These cements, besides being moldable into bone interstices and capable of hardening *in situ* with minimal release of residual monomer, are biodegradable with a controlled rate of resorption leading to eventual ingrowth and replacement by new bone. They can provide initial structural support, exhibiting mechanical properties within the range of normal trabecular bone (5–12 MPa compression strength and 0.02–0.50 GPa Young's modulus), and also provide sufficient rigidity and strength during degradation. Ideally, the degradation rate should couple to the rate of tissue formation, and the amount of void space should be adequate to allow tissue vascularization. Potential uses for such biodegradable cements include structural reinforcement of age-related fractures[1] or as an artificial bone substitute.[2]

Another important application of self-polymerizing composites is focused on the field of controlled delivery systems. In orthopedics, PMMA beads and antibiotic-impregnated acrylic bone cements have been used in the treatment of chronic osteomyelitis and other musculoskeletal infections for many years[3] due to the inefficiency of the systemic administration. Parenteral administration of antibiotics is an inefficient method to achieve a high local tissue drug concentration, partially due to the poor blood circulation of the osseous tissue. If high systemic levels are maintained, then there is a potential risk for organ toxicity such as hearing and kidney damage. However, as poly(methyl methacrylate) beads are inert, they must be removed after approximately 2 weeks, otherwise bacteria can adhere and grow on their surface and potentially develop antibiotic resistance. As an example of this, an exceptional case was found in which these beads were left *in situ* for 5 years. Studies carried out on these beads revealed residual antibiotic release after 5 years *in situ*, and a gentamicin-resistant staphylococcal strain was recovered from the surface of the beads.[4] This case emphasizes the importance of developing biodegradable antibiotic-loaded beads as antibiotic delivery systems. The biodegradable cements[5] have the advantage of releasing their entire load of drug as they degrade, in contrast to the limited amount that can be diffused out of an inert matrix such as that of PMMA.

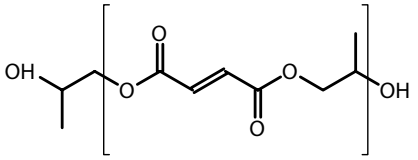
4.2 COMPOSITES BASED ON POLY(PROPYLENE FUMARATE)

Poly(propylene fumarate) (PPF), an unsaturated linear copolyester that can be crosslinked through the fumarate double bond, has been used to prepare injectable and degradable composites. The degradation products of PPF are fumaric acid, a naturally occurring substance, found in the tricarboxylic acid cycle (Kreb's cycle), and 1,2-propanediol, which is a commonly used diluent in drug formulations. Figure 4.1 shows the chemical structure of PPF. It appears that achieving high-molecular-weight PPF is difficult because of side reactions, particularly due to the presence of the backbone double bond. Accordingly, incorporation of fillers or further reactions to form crosslinked networks would be required to achieve good mechanical strength.

This polymer has been crosslinked with monomers such as methyl methacrylate (MMA) or *N*-vinyl pyrrolidone (*N*-VP) (Figure 4.1). These systems are based on two components, a solid and a liquid phase, and the crosslinking reaction is initiated by an amine/peroxide redox system, in which benzoyl peroxide is the initiator and *N,N'*-dimethyl-4-toluidine is the activator. The polymerization of the material itself can be adjusted to cause foaming through the release of carbon dioxide, thereby producing a porous scaffold, as seen in Figure 4.2.[6]

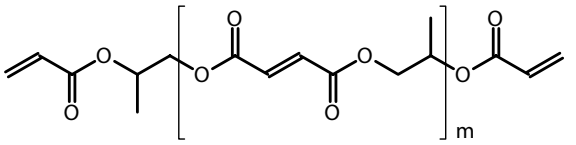
Composite bone cements consisting of tricalcium phosphate (TCP) and calcium carbonate in a matrix of poly(propylene fumarate) crosslinked with MMA have been prepared for use in different applications such as structural reinforcement of osseous defects, internal fixation devices, and delivery of antibiotics for osteomyelitis treatment.[7] The properties of the cements and the degradation rates depended on the concentration of the reactants.[8] An increase in MMA or both MMA and BPO content was inversely proportional to the rate of degradation and directly proportional to

Poly(propylene fumarate), PPF



Methyl methacrylate, MMA N-vinyl-2-pyrrolidone, N-VP

Poly(propylene fumarate)-diacrylate, PPF-DA, m=1,2



Poly(ethylene glycol)-dimethacrylate, PEG-DMA

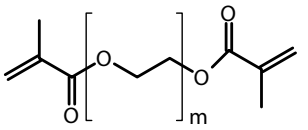


FIGURE 4.1 Chemical structure of poly(propylene fumarate) and some of the monomers used to obtain crosslinked composites.

the initial mechanical properties. For example, the formulation consisted of one-third PPF/MMA, one-third calcium carbonate, and one-third tricalcium phosphate presented initial compressive strengths of up to 30 MPa and compressive modulus of up to 300 MPa. The process of cement degradation was found to be similar to physiological bone remodeling.[9]

The mechanical properties and degradability of PPF cements are mainly influenced by the polymer molecular weight and polydispersity, and by the polymer end-groups. In order to study these factors, different formulations of cements were prepared from 70-wt% calcium salts and a liquid phase of PPF oligomers.[10] In some cases, MMA and *N*-vinyl pyrrolidone were used to determine the effect of the monomers on the crosslinking ability of the compositions. The PPF terminated in divinyl groups presented the highest compressive strength. The strongest cement was that whose matrix consisted of the divinyl-derivative oligomer crosslinked with both MMA and *N*-VP monomers, followed by that crosslinked only with MMA. Those composites based on diol- or epoxide-terminated PPF and containing diluents such as methyl pyrrolidone or ethyl lactate were the weakest materials. The other important parameter is the degradation rate. The composites crosslinked with MMA produced a tight crosslinked polymer matrix that showed less accessibility to water. On the contrary, the formulations prepared in absence of monomers formed

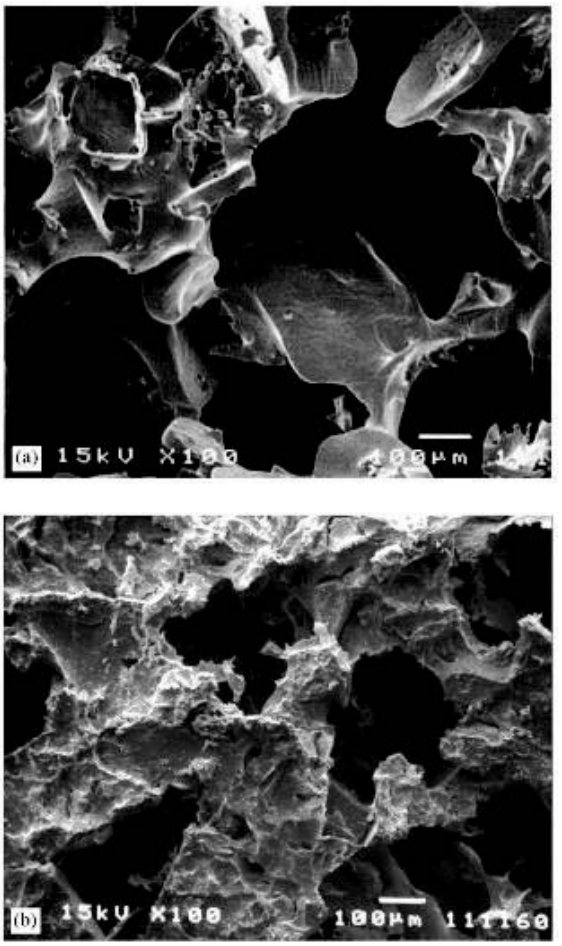


FIGURE 4.2 (a) Scanning electron micrograph of PPF scaffold: 10-wt% PPF, 90-wt% porogen; porogen removed with water leaching after PPF crosslinking. (b) Scanning electron micrograph of PPF scaffold: 30-wt% PPF, 70-wt% porogen; porogen removed with water leaching after PPF crosslinking. (From Burg, K.J.L., Porter, S., and Kellam, J.F., *Biomaterials*, 21, 2347, 2000. With permission.)

a loose matrix that was more accessible to water penetration and hydrolysis. The specimens composed of epoxide-terminated PPF oligomers showed the least degradation and water absorption compared with those based on the diol- and divinyl-terminated PPF, which showed similar behavior between themselves. There was a good correlation between the compressive strength losses and the hydrolytic degradability.

The crosslinking characteristics of the injectable composite based on PPF, *N*-vinyl pyrrolidinone (*N*-VP), sodium chloride, and β-tricalcium phosphate (β-TCP) have been investigated by varying the PPF molecular weight, *N*-VP/PPF and BPO/PPF ratios, and NaCl content in the formulation.[11] Heat release increased with decreasing molecular weight of PPF and increasing content of *N*-VP and NaCl, but in any case, the highest maximum temperature reached during the crosslinking reaction was around 50ºC lower than those presented by PMMA. The gel point was most affected by the PPF molecular weight and the amount of BPO. By altering the composite formulation, the gel point could be varied from 1 min to 2 h. In general terms, mechanical properties were close to those of human trabecular bone and increased with a decrease in *N*-VP content and an increase in both NaCl and BPO concentrations in the formulation, but they are expected to decrease after *in vivo* implantation due to dissolution of the porogen salt in the physiological fluids.[12] These com-

posites can be used to fill skeletal defects, acting both as a scaffold for the cell growth and as a mechanical support at the defect site.

Injectable PPF/*N*-VP cements with osteoconductive properties were prepared with calcium gluconate/hydroxyapatite (HA) as fillers.[13] This filler combination including both soluble and insoluble components was selected due to its respective ability to develop porosity and osteoconductivity, requirements which are essential to support bony ingrowth. The *in vivo* evaluation in rat showed dissolution and leaching of the soluble calcium salts, resulting in cavitations filled with vascular and bony tissue. Likewise, active new endosteal bone remodeling with deposition of osteoid at the cement–bone interface in the proximal and distal femoral diaphysis and metaphysis confirmed the osteoconductivity of this injectable formulation. Further resorbable composites with osteoconductive properties were formulated with PPF/*N*-VP and HA as bioactive filler in the presence of effervescent agents to yield a porous microstructure upon curing. These systems are designed to be used as porous bone graft substitutes and fracture fixative.[14] One potential application of this material is the stabilization of metacarpal or phalangeal fractures to augment conventional fixation techniques. After mixing both liquid and solid phases, the reaction of the effervescent agents, citric acid, and sodium bicarbonate resulted in expansion of the graft material, producing pore sizes of 50–500 μm after curing, which are within the desirable range for optimal bone cell ingrowth (150–500 μm).[15] Mechanical properties of these systems were evaluated during *in vitro* degradation. Initial mechanical properties of the resorbable system compared well with those of cancellous bone, and the material lost compressive strength at a rate of approximately 0.5 MPa per week as a result of degradation, coinciding with the expected strength recovery of healing bone. The efficiency of the material for stabilization of fractures was assessed in an intramedullary nailing model. Osteotomized femora were stabilized with and without the composite, and fracture fixation was evaluated by three-point bending. It was found that the addition of PPF-based material significantly improved the flexural strength (31 ± 7% of intact bone) versus femora fixed only with threaded K wire (11 ± 2% of intact bone) as used clinically.

Polymeric networks of poly(propylene fumarate) crosslinked with the macromer poly(propylene fumarate)–diacrylate (PPF-DA) have been prepared due to concern regarding the toxicity of the unreacted monomer vinyl pyrrolidone and the formation of nondegradable poly(vinyl pyrrolidone) (PVP) links.[16] On the contrary, the PPF/PPF-DA network degrades into biocompatible constituents consisting mainly of fumaric acid, propylene glycol, and poly(acrylic-co-fumaric acid) with an average molecular weight around 5.000. A wide range of mechanical properties can be obtained by varying the double bond ratio of PPF/PPF-DA and the molecular weight of PPF-DA. It was observed that a decrease of the double bond ratio from 4 to 0.5 provided an improvement of the compressive strength from 11.2 ± 1.8 to 66.2 ± 5.5 MPa, and an increase of molecular weight of PPF-DA caused an increase of compressive strength up to 88.2 ± 6.1 MPa. The degradation of the networks can be controlled by the crosslinking density and prolonged with the incorporation of the β-TCP filler. *In vitro* studies[17] showed an initial improvement of mechanical properties in the first 12 weeks, which has been attributed to the crosslinking of unreacted bonds within the networks. From the initial strengthening, the materials maintained their properties until a sudden decrease in the fracture strength was measured after 52 weeks. This reduction was accompanied by a bulk degradation behavior conducive to the release of acidic products, mainly fumaric acid. A decrease in PPF/PPF-DA crosslinking density reduced the initial mechanical properties, but increased the degradation rate.

Recently, the preparation of the composites PPF/PPF-DA has been carried out by photopolymerization using a new molding process.[18] The molds were transparent silicone molds formed by curing room-temperature vulcanizing silicone over a master pattern suspended in an open container. This kind of mold offers the ability to cast photocurable resins that will be activated with light exposure through the mold wall; the specimens of this work were cured at 100ºC for 24 h to ensure complete crosslinking. There was a general trend of increasing shrinkage with increasing double bond ratio (DBR), reaching a value of around 12% for a PPF/PPF-DA double bond ratio of 2. In

general, the mechanical properties of the so-obtained specimens increased with decreasing DBR as observed in previous studies. The increased PPF-DA content at the lower DBR enhances the crosslinking reaction, yielding a greater crosslinking density. The compressive moduli and fracture strengths over the same DBR range were 310–1270 MPa and 60–130 MPa, respectively. The improved properties of these materials with respect to those previously photopolymerized in glass molds and UV light[19] can be attributed to a higher light density for photocrosslinking and the postcuring of the specimens. Mechanical properties increased significantly with gamma sterilization. Compared with other biodegradable polymers such as poly(*L*-lactic acid) (PLLA), a common biodegradable orthopedic implant, the PPF/PPF-DA networks present similar mechanical properties.

For applications such as the engineering of softer orthopedic tissues such as cartilage, composites based on PPF crosslinked with poly(ethylene glycol)-dimethacrylate (PEG-DMA) (Figure 4.1) and containing β-tricalcium phosphate as the filler are suitable.[20] These cements present maximum temperatures averaging 39.7ºC and gel points varying from 8 to 12.6 min. Mechanical properties in compression of the polymer networks and crosslinked composites increased with the PEG-DMA/PPF double bond ratio, although they decreased with water absorption due to the hydrophilicity of PEG. The compressive strength for dry materials increased from 5.9 ± 1.0 to 11.2 ± 2.2 MPa as the double bond ratio of PEG-DMA/PPF increased from 0.38 to 1.88. However, in the wet state, the compressive strength at yield was in the range of 2.2 ± 0.5 to 3.5 ± 0.5 MPa. The addition of β-TCP enhanced the mechanical properties, with the compressive strength ranging between 41.4 ± 1.0 and 76.0 ± 1.3 MPa.

4.2.1 Antibiotic Delivery Systems

Formulations consisting of tricalcium phosphate and calcium carbonate dispersed in a matrix of PPF crosslinked with MMA have been assayed as carrier agent for local release of antibiotics, and they achieved higher sustained local levels and serum levels of antibiotics in comparison with those obtained by PMMA cement. Recently, Sanderson[21] developed the preparation of biodegradable bone cements crosslinked with *N*-VP; the cements were employed as carriers for controlled drug release, where drug release and polymer degradation occurred at equivalent rates, and rates were proportional to surface area, indicating that the release of the drug is controlled by surface erosion rather than by diffusion. Further studies on the reaction of *N*-VP with PPF revealed that the amount of *N*-VP incorporated into the crosslinked structure depended on the PPF/*N*-VP ratio, and when this ratio was unity, only about 50% of the *N*-VP was incorporated.[22]

4.3 COMPOSITES BASED ON POLY(METHYL METHACRYLATE)

Partially degradable systems based on poly(methyl methacrylate) have been prepared by using a solid phase consisting of poly(methyl methacrylate) beads or a liquid phase composed of methyl methacrylate or other soluble monomers. In general terms, these types of systems can be applied as bone substitutes in fracture repair and in drug delivery systems.

4.3.1 Resorbable Components in the Solid Phase

In situ curing cement suitable for tissue ingrowth has been developed, dispersing an aqueous gel based on carboxymethyl cellulose into a paste of a traditional formulation of acrylic bone cement. The aqueous phase and the acrylic phase were mixed in a ratio of 1:1 by weight.[23] The addition of the gel phase produced a reduction of the maximum temperature reached during the curing of the acrylic resins, which did not exceed 60ºC. When the cement was placed in a biological environment, the gel dissolved, leaving a pore distribution in the cured material with pores 300–1000 μm in diameter, which is optimum for hard tissue to grow and vascularize inside. Mechanical properties were drastically influenced by the presence of pores, and the porous cement possessed lower strength and stiffness. Tensile strength of 50% porous cement was about 3 MPa, and elastic

modulus was about 300 MPa. This indicates that the clinical application of these materials should be confined to areas in which high loads are not expected. The hard tissue reaction to the implantation of this cement revealed the ingrowth of fibrous tissue in the pores formed by the dissolution of the gel. The original bone surrounding the implant was bioresorbed, creating a soft tissue interface. Then, a front of bone deposition appeared, following the direction of the implant, and after 6 weeks, substantial bone ingrowth was found, anchoring the implant to the host bone. After 1 year, about 75% of the available pore volume was filled.

Partially resorbable polymeric composites with bioactive properties have been prepared by adding aqueous α-TCP dispersions to a PMMA bone cement.[24] The final structure of the composite was a polymeric porous body with the bioactive inorganic phase confined inside the pores and not bonded to the polymer as confirmed by SEM analysis. The resulting composite presents microstructure and mechanical properties suitable for application as bone substitutes. These formulations had longer setting times and lower peak temperatures than those of PMMA. The mechanical properties of the obtained porous composites have values comparable to those of porous bioceramics of HA currently used as bone substitute compounds. The biological evaluation of this cement was conducted on osteoblast cultures that were stimulated with pulsed electromagnetic fields. The results showed an improvement in proliferation and synthetic activity of the PMMA/α-TCP cement.[25]

Other resorbable acrylic bone cements have been prepared by incorporation of an experimental oligomer filler consisting of the polyamide amino acid *trans*-4-hydroxy-*L*-proline, in amounts varying between 5 and 20 wt%.[26] These systems were based on the aforementioned hypothesis that the addition of the resorbable oligomer would cause porosity formation in the cement in the physiological medium due to sorption of water. When samples were conditioned in water or simulated body fluid solution (SBF), an average pore size between 10 and 500 μm was obtained from SEM micrographs, and pores were randomly distributed in the matrix. The hydrolysis of the oligomer could have occurred during its dissolution, and this could have increased the velocity of the pore formation. The amount of oligomer filler was not large enough to get a uniform pore distribution in the entire volume of the specimens, but it was the maximum admitted in order to maintain the traditional acrylic bone cement formulation. The porosity obtained decreased both flexural and compressive strengths, but no significant difference was found between the storage of specimens in water or SBF. The compressive strength of the cement containing 20 wt% of the oligomer filler in the dry state was 102 MPa, even higher than that corresponding to plain PMMA, but after 1 week of immersion in SBF, the compressive strength decreased to 61 MPa. Accordingly, flexural strength for the same specimen decreased from 37 to 20 MPa, corresponding to the dry state and to 1 week of storage in water.

Composite and partially degradable starch/cellulose acetate/hydroxyapatite (SCA/HA) acrylic bone cements with controlled balance of hydrophobicity have been formulated from a processed blend of starch and cellulose acetate (SCA) and different mixtures of acrylic monomer, acrylic acid (AA), and MMA, in the presence of HA.[27] The kinetic parameters of the curing formulations activated at low temperature allow the selection of the optimum solid/liquid (S/L) ratio and the composition of hydrophilic/hydrophobic (AA/MMA) components of the most appropriate formulations. The best results in terms of curing parameters and mechanical properties were those with S/L ratio 55/45 (as seen in Figure 4.3), a content of HA of about 20 wt%, and a best hydrophobicity balance with a composition of 38% MMA and 7% AA. The heterogeneous morphology of the cured cements can be positively applied for the formation of a relatively porous material with a compensation of the contraction of volume after the polymerization and the induction of bioactivity by the presence of HA and biodegradability by the presence of SCA.

4.3.2 Resorbable Components in the Liquid Phase

Partially biodegradable composites with ferro- and piezoelectric properties have been developed for use in fracture repair of long bones. The composites were formulated with hydroxyapatite-

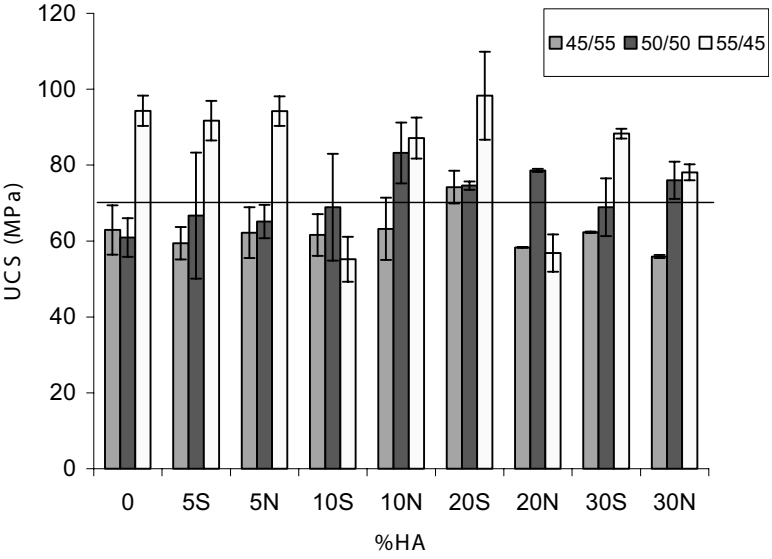


FIGURE 4.3 Comparing maximum compressive strength for all prepared bone cement formulations. Black line marks the minimum value for ASTM specifications (UCS: Ultimate Compressive Strength; N: nonsintered HA; S: sintered HA). (From Espigares, I. et al., *Biomaterials*, 23, 1883, 2002. With permission.)

coated barium titanate granules and PMMA beads dispersed in a hydrophilic matrix of poly(methyl methacrylate-co-vinyl-2-pyrrolidone).[28] The rationale behind this formulation was that the partial biodegradability of the matrix would permit the osteointegration of the implant and the ceramic filler would generate the desired *in situ* electrical stimulation, which could contribute to restore the cathodic potential in the zone of the fracture.[29] When these cements were placed in a simulated body fluid, they absorbed water up to a hydration degree ranging between 2 and 16%, depending on the amount of filler, and they experimented weight loss due to the partial dissolution of the matrix. The presence of VP monomer also slowed down the polymerization rate with respect to that of plain PMMA, providing a decreasing of the setting time and also producing a lowering of the maximum temperature, which reached levels around that of the physiological temperature. Regarding mechanical properties, the addition of VP produced a reduction of the compression properties of the cement, but this effect was compensated for with the introduction of the filler in amounts higher than 30 vol%, and the composites presented values of compressive strength around 80 MPa.

Partially resorbable composite materials were prepared based on the Bowen molecule 2,2-*bis*(*p*-[2′-hydroxy-3′-methacryloxypropoxy]phenyl)propane (*bis*-GMA) and the diluent TEGDMA by crosslinking copolymerization with oligo(lactone) macromonomers in the presence of hydroxyapatite (45% m) as a bioactive filler. This material should enable the ingrowth of bone after a defined time. The *in vivo* response to the implantation of *ex vivo* cured rods of this material in rabbits after 24 weeks was analyzed by SEM and SFM techniques, and it was observed that bone growth was directed toward hydroxyapatite particles and agglomerates after partial degradation of the polymer matrix, and the gaps generated between particles and the matrix were filled with mineralized tissue.[30]

4.3.3 Antibiotic Delivery Systems

As stated in the previous section, one of the main applications of partially biodegradable systems is to act as carriers for the controlled delivery of drugs. However, considering the physicochemical characteristics of self-curing PMMA-based acrylic formulations when bulk filling mass or PMMA balls are charged with different kinds of drugs like antibiotics, analgesics, or anti-inflammatory

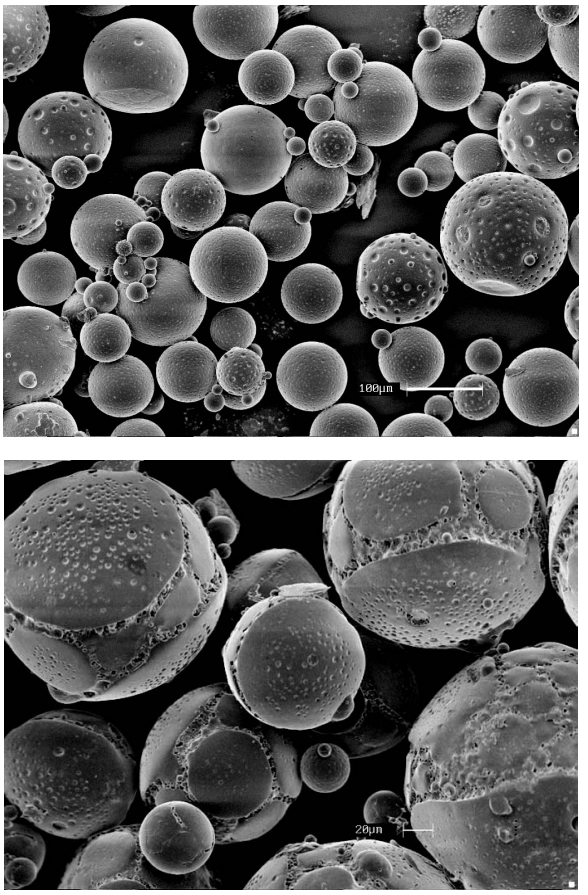


FIGURE 4.4 SEM pictures of PMMA/PCL beads (top) 89:11; (bottom) 83:17. (From Mendez, J.A. et al., *J. Biomed. Mater. Res.*, 61, 66, 2002. With permission.)

drugs, the release of these compounds is mainly restricted by the low permeability of the PMMA-based formulations to the physiological fluids. This phenomenon justifies the design and application of new formulations with components that are not chemically stable in physiological conditions, i.e., poly(ε-caprolactone) or an alternative way based on compounds that are soluble in the physiological medium such as soluble phosphate glasses. Based on the first alternative, partially biodegradable acrylic cements have been formulated with poly(methyl methacrylate)–poly(ε-caprolactone) (PMMA/PCL) beads, which were obtained by suspension polymerization of MMA in the presence of PCL.[31] Beads of PMMA/PCL of 89:11 or 77:23 weight ratio were mixed with MMA in a solid:liquid (s:l) ratio of 1.5:1 to prepare the cements (Figure 4.4).[32]

The physical and chemical microheterogeneity of these beads influenced significantly the curing parameters because several aspects involved in the polymerization reaction are closely related to both morphology and size distribution of the particles. Because of the very slow degradation rate of PCL, a small decrease of the fraction of this compound was observed with time after immersion of samples in SBF. Only approximately 2-wt% weight loss was measured after a period of 8 weeks. Mechanical properties of these composites decreased with the content of PCL with respect to PMMA, due to the introduction of a semicrystalline polymer with poor mechanical properties. However, the compression properties for specimens soaked in SBF for 4 weeks were higher than those of trabecular bone, which presented values of compression strength varying from 69 to 47 MPa and those of elastic modulus ranging from 1138 to 869 MPa. This means that this type of

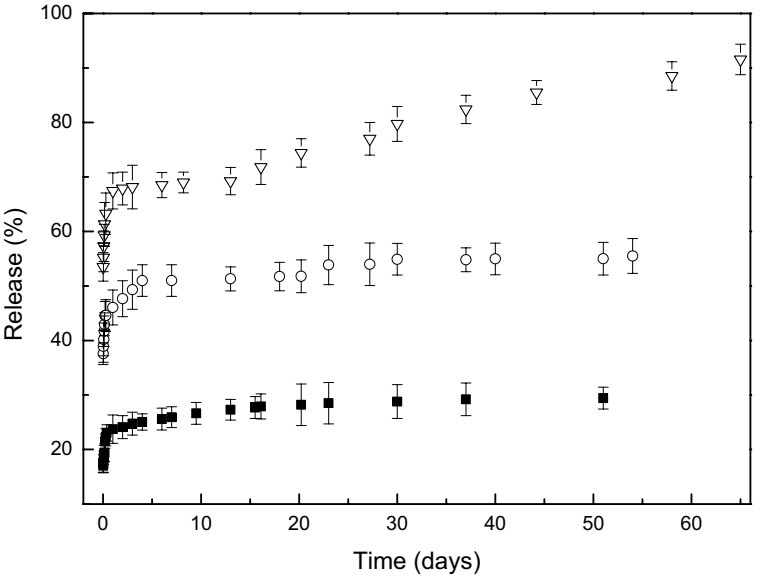


FIGURE 4.5 Vancomycin release profiles of samples with different PMMA/PCL ratio: (■) 100/0 (control), (▽) 86/14 (s:l = 1.5:1), (○) 77/23 (s:l = 1.3:1). (From Mendez, J.A. et al., *J. Biomed. Mater. Res.*, 61, 66, 2002. With permission.)

system can provide mechanical support after implantation. These composites were effective as carriers for local release of vancomycin, showing an initial burst release of the drug in the first 5 h and then a sustained release over a period of 2 months, in which the total amount of vancomycin released (between 64 and 90%) depended on the composition of the PMMA/PCL beads (Figure 4.5). The composite prepared with beads that had a PMMA/PCL ratio of 86:14 released nearly the total amount of the initial drug (90%) in approximately 2 months.

Composites based on PMMA and soluble phosphate glasses in the system P_2O_5-44.5, CaO-44.5, Na_2O-11 (mol%) were also prepared for use as carriers of antibiotic controlled delivery.[33] These glasses have a chemical composition similar to that of the inorganic phase of bone and are completely soluble in aqueous medium. The aim of adding the soluble glasses was to contribute to the diffusion of the drug as the dissolution of the glass in the physiological medium takes place. The introduction of this inorganic compound in amounts of 30–70 wt% in the solid phase provides fluid pastes after approximately 10 min, which permitted their injection into the corresponding cavity. In addition, the maximum temperature reached during the curing of these composites was in the range of 40–60ºC, which was much lower than that of PMMA, diminishing the risk of thermal and chemical trauma at the site of implantation. The elution of vancomycin from these composites presented a moderate initial burst release in the first 30 min, followed by a sustained release in a period of 40 days, with 60 and 94% of initial drug released for the composites containing the highest amounts of phosphate glasses. The elution profile was correlated with that of weight loss obtained after soaking the composites in SBF. However, contents of phosphate glasses around 30 wt% in the solid phase did not improve the vancomycin release with respect to PMMA formulations.

Systems based on corn starch–cellulose acetate blends obtained by the free-radical polymerization of methyl methacrylate or acrylic acid in the presence of the carbohydrates[34] also have potential use as carriers in drug delivery systems since they are sensitive to pH and possess a clear reversible transition in a relatively narrow interval of pH, just in the range of physiological conditions.

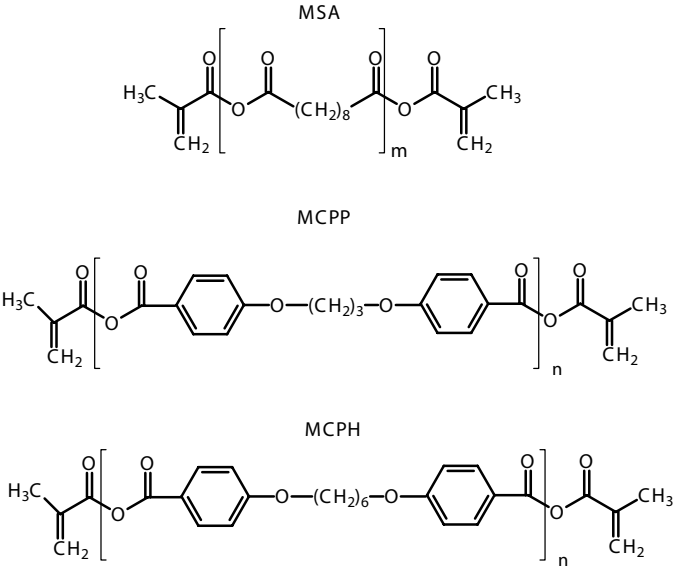


FIGURE 4.6 Methacrylated anhydride monomers of sebacic acid (MSA), 1,3-*bis*(*p*-carboxyphenoxy)propane (MCPP), and 1,6-*bis*(*p*-carboxyphenoxy) hexane (MCPH).

4.4 PHOTOPOLYMERIZED SYSTEMS

Degradable injectable systems have been developed from methacrylated anhydride monomers of sebacic acid and 1,6-*bis*(carboxyphenoxy) hexane, which can be photopolymerized using ultraviolet light, giving rise to highly crosslinked polyanhydride networks (Figure 4.6).[35]

Mechanical properties were governed by the crosslinking density of the resulting polymer network, while degradation rate was controlled by the chemical composition, which can vary from 2 days to 1 year. Another route to control the degradation rate was the formulation of semi-interpenetrating polymer networks (semi-IPN), which were obtained by combining the methacrylated anhydride derived from sebacic acid with a linear copolymer of 1,3-*bis*(carboxyphenoxy) propane (CPP) and 1,6-*bis*(carboxyphenoxy) hexane (CPH). Once the anhydride is polymerized, the fragments of the linear copolymer would hinder the penetration of water, increasing the hydrophobicity of the final network. The erosion rate was significantly reduced compared with the crosslinked homopolymer. The slope of the degradation curves varies by more than a factor of 30. These systems were evaluated in terms of mechanical properties to be used in fracture fixation applications. In general, mechanical properties were intermediate between those of trabecular and cortical bone. Mechanical properties were evaluated during the degradation of the crosslinked network, and it was found that the systems maintained their structural integrity and more than 70% of their tensile modulus at 50% of mass degradation. This is advantageous with respect to other biodegradable systems. The crosslinkable systems provide flexibility in the first stage, and then they enhance mechanical properties as the resulting polymer degrades. The biocompatibility of the polyanhydrides was studied by subcutaneous implantation in rats during a period of 28 weeks, and after this time, loose vascularized tissue was found in the implant, but there was no evidence of fibrous capsule formation.[36]

Photocured dual-setting cements based on α-tricalcium phosphate (α-TCP) as solid component and a mixture of soluble acrylic monomers, acrylamide, and *N,N'*-methylenebisacrylamide as liquid component have been prepared for use as dental composites.[37] In this new dual-setting technique, two setting reactions proceed after mixing the powder and the liquid components. One is a

conventional hydraulic reaction to obtain a calcium-deficient hydroxyapatite, and the other one is the photopolymerization of the acrylic monomers. The resulting material is a network composed of an acrylic matrix in which the calcium-deficient hydroxyapatite crystals are dispersed. This material improved significantly some of the important properties of the conventional calcium phosphate bone cements.

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5 Fiber Bonding and Particle Aggregation as Promising Methodologies for the Fabrication of Biodegradable Scaffolds for Hard-Tissue Engineering

Manuela E. Gomes, Patrícia B. Malafaya, and Rui L. Reis

CONTENTS

5.1 INTRODUCTION

One of the most widely studied tissue engineering strategies for the creation of hard-tissue (such as bone and cartilage) substitutes relies on the use of a temporary 3-D scaffold material within which cells are seeded and cultured *in vitro* prior to implantation.

In this type of strategy, the formation of new tissue is deeply influenced by the three-dimensional environment provided by the scaffolds, namely its composition, porous architecture, and, of course, its biological response to implanted cells and surrounding tissues. In order to meet all the necessary requirements, scaffold materials must be fabricated from polymers with adequate properties. However, the establishment of basic requisites for scaffolds associated to its design constraints is not an easy task and requires a deep knowledge of all the material features that can interfere with cells/tissues–scaffold interactions. Many of these features are dictated by the processing methodology used to fabricate the scaffolds. The development of matrices to serve as templates for cell attachment/suspension and delivery has progressed at a tremendous rate in recent years, and a wide range of methodologies has been developed. Among these processing techniques are methods such as solvent casting and particulate leaching,[1–6] membrane lamination,[1,7] fiber bonding,[8–14] phase

separation/inversion,[1,15] melt-based technologies,[3,16–19] microparticle aggregation,[20,21] and microwave baking and expansion,[22] just to cite some examples. More recently, highly reproducible 3-D scaffolds have been obtained using rapid prototyping technologies such as fused deposition modeling (FDM) and 3-D printing.[8,23–25]

Many of these technologies fail to provide the scaffolds with basic requirements such as pore interconnectivity and suitable mechanical properties, and most of them are only able to produce scaffolds with specific requirements for a limited number of applications.

Fiber bonding and aggregation of microparticles are two very distinct processing methodologies that might enable one to circumvent important limitations of most scaffold fabrication methods and produce scaffolds with unique features that enable their use in many different hard-tissue engineering approaches, as will be discussed later in this chapter.

5.2 REQUIREMENTS FOR TISSUE ENGINEERING SCAFFOLDS AND SCAFFOLD FABRICATION TECHNIQUES

Besides the obvious demands of biocompatibility and biodegradability, an ideal tissue engineering scaffold should exhibit appropriate mechanical properties[19,26–30] and a suitable degradation rate.[8,19,27,29–31] Furthermore, the scaffold must possess adequate porosity, interconnectivity, and permeability to allow the ingress of cells and nutrients,[8,19,29,30] as well as the appropriate surface chemistry for enhanced cell attachment and proliferation.[29,30,32,33] For most applications, tissue engineering scaffolds must provide cell anchorage sites, mechanical stability, and structural guidance and, when implanted, provide an adequate interface to respond to physiological and biological changes in order to integrate with the surrounding native tissue.

Taking into account the aforementioned requisites, the processing technology used to produce the scaffolds is mainly expected to provide the maximum control over macro- and microstructural properties of the scaffold without negatively affecting the other properties that provide good scaffold–cell interactions such as toxicity and surface chemistry or topography.[34] Other important requirements for a scaffold fabrication method include process accuracy and reproducibility — i.e., the methods should be able to produce scaffolds with consistent properties and physical forms when using the same processing parameters.

However, the properties of the scaffold are also dependent on the natural or synthetic material selected for its production. For the selection of the most appropriate scaffold material for a given application, besides the intrinsic properties of the wide range of materials available, it is also important to consider that each material or combination of materials has a different degree of processability and processing requirements.

The methodologies developed so far have been applied to the fabrication of scaffolds for tissue engineering with different levels of success. Usually, these methods involve either melting or solvent-casting processing. Melt processing involves heating the polymer above the glass transition temperature (T_g) or the melting temperature (T_m) and depends on melt viscosity. Solvent processing depends on polymer solubility in various organic solvents and on the solvent volatility.[31,35] In general, most of these methods present the following main limitations:[34]

1. *Manual intervention:* Most of the techniques available rely on manual-based procedures that are practically impossible to transform in industrial scaled-up methods. Therefore, the processes become labor intensive and time consuming. Furthermore, the process is very dependent on the user's skill, and the reproducibility of results is very difficult to achieve.
2. *The use of toxic organic solvents:* The use of toxic solvents in technologies based in the casting of polymeric solutions may affect the biocompatibility of the resulting scaffold due to retention of harmful residues.
3. *Use of porogens:* The methods based on porogen leaching are usually limited to the production of thin scaffolds to facilitate the total removal of the porogens, which can

have a cytotoxic effect on the contacting cells. Furthermore, it is difficult to avoid the agglomeration of the porogen particles, leading to non-uniform porosity and pore size.
4. *Geometric limitations:* Several techniques use molds or containers to cast scaffolds that only allow for thin membranes and basic, uniform scaffold geometries.

Nevertheless, one of the major problems of the scaffolds produced by most of the methods currently available is their poor mechanical properties and inadequate porosity. Obviously, it is very difficult to optimize these two properties simultaneously, since the mechanical properties usually decrease with increasing porosity. However, these are very important aspects for the regeneration of hard tissues because scaffolds are required to provide structural support for the neo-tissues, and porosity and interconnectivity of the structures are essential to allow for cell proliferation within constructs with significant sizes. In addition, the complexity of the problems that need to be solved in regenerative medicine should be reduced by using material scaffold designs that allow them to perform multiple functions. Therefore, the search for better methods of producing porous scaffolds so that physical and chemical properties can be simultaneously optimized is still an important and challenging issue, especially in hard-tissue engineering research.

5.3 FIBER BONDING

5.3.1 Fiber Meshes

Fiber meshes consist of individual fibers either woven or knitted into three-dimensional patterns of variable pore size.[1,3,31,32,35]

The most important advantageous features of scaffolds obtained by fiber-bonding processes, i.e., fiber meshes, are a large surface area for cell attachment and a rapid diffusion of nutrients, which enhances cell survival and growth.[1,3,31,32,35] This, of course, results from a high interconnectivity among pores. A drawback of these scaffolds might be the difficulty in accurately controlling the porosity.[1,3,31,35]

Several studies demonstrate that scaffolds obtained by fiber-bonding processes have adequate structure for use in tissue engineering strategies that use bioreactor cultures, probably because they provide highly interconnected porosity that enables the creation of hydrodynamic microenvironments with minimal diffusion constraints that closely resemble natural interstitial fluid conditions *in vivo*, allowing the development of large and well-organized cell communities. On the contrary, most of the pores obtained with other methodologies exhibit lower interconnectivity, which is very likely to generate complex fluid-flow pathways through the scaffolds and does not allow for the distribution of cells throughout the whole construct.

Fiber-bonding methods include a great variety of processing methods that involve the knitting or physical bonding (by means of casting or compression procedures) of fibers prefabricated by wet or dry spinning from polymeric solutions or by melt spinning. Fiber meshes may also be obtained in single-step methods such as electrospinning.

Polyglycolic acid (PGA) nonwoven meshes have been widely used in tissue engineering studies, particularly concerning applications in cartilage reconstruction.[13,14,33,36–40] These meshes are produced by extrusion of PGA into 13-μm-diameter fibers, which are subsequently stretched and relaxed at high temperatures, crimped and cut, carded into a lofty web, and finally needled to form a nonwoven mesh. Theses meshes are commercially available and are produced by Albany International (Mansfield, MA).[33]

Freed et al.[13,14,33,36–40] have performed several studies where they used these PGA fiber meshes and freshly isolated chondrocytes to investigate the influence of scaffold properties as well as seeding and culture methods on the development of cartilage substitutes. For example, in one of those studies,[14] these highly porous PGA scaffolds, 5 to 10 mm in diameter and 2 to 5 mm thick, were seeded with bovine articular chondrocytes in spinner flasks to investigate optimal seeding

conditions. Essentially all cells attached throughout the scaffold volume within 1 day. The mixing provided by the spinner flasks promoted the formation of 20- to 32-micron-diameter cell aggregates that enhanced the kinetics of cell attachment without compromising the uniformity of cell distribution. The kinetics and possible mechanisms of cell seeding were related to the formation of cell aggregates by a simple mathematical model that can be used to optimize seeding conditions for cartilage tissue engineering. The same types of scaffolds and cells were cultured in a rotating bioreactor system to assess the effect of culture conditions.[13] It was found that concomitant increases in the amounts of glycosaminoglycan (GAG) and type II collagen resulted in cell–polymer constructs with continuous cartilaginous matrix over their entire cross sections (6.7 mm diameter × 5 mm thick) after 40 days of cultivation. Compared with natural calf cartilage, constructs had comparable cellularities, 68% as much GAG, and 33% as much type II collagen per gram (net weight). The progression of chondrogenesis in chondrocyte–polymer constructs was similar to that suggested previously for precursor cells *in vitro* and developing limbs *in vivo*. In particular, the polymer scaffold provided a three-dimensional structure that could be seeded with chondrocytes at high cell densities in order to establish cell-to-cell contacts and initiate cartilage tissue development, whereas the bioreactor vessel provided a permissive microenvironment for chondrogenesis.

In another work,[40] the PGA scaffolds were seeded with bovine calf articular chondrocytes and then cultured in three different environments (static flasks, mixed flasks, and rotating vessels) to assess the relationships between the composition and mechanical properties of engineered cartilage constructs. After 6 weeks of cultivation, the composition, morphology, and mechanical function of the constructs in radially confined static and dynamic compression all depended on the conditions of *in vitro* cultivation. Static culture yielded small and fragile constructs, while turbulent flow in mixed flasks yielded constructs with fibrous outer capsules; both environments resulted in constructs with poor mechanical properties.[40] The constructs that were cultured while freely suspended in a dynamic laminar flow field in rotating vessels were the largest, contained continuous cartilage-like extracellular matrices with the highest fractions of glycosaminoglycan and collagen, and had the best mechanical properties. The equilibrium modulus, hydraulic permeability, dynamic stiffness, and streaming potential correlated with the wet-weight fractions of glycosaminoglycan, collagen, and water. These findings suggest that the hydrodynamic conditions in tissue-culture bioreactors can modulate the composition, morphology, mechanical properties, and electromechanical function of engineered cartilage. This work[40] demonstrates the promise of using PGA fiber meshes for the production of tissue-engineered constructs for the reconstruction of cartilage using dynamic *in vitro* culturing systems.

Other researchers[41] have investigated the possibility of creating cartilage *in vitro* using two resorbable nonwoven cell scaffolds, with different degradation characteristics, based on polyglycolic acid/poly-*L*-lactic acid (PGA/PLLA) copolymer (Ethisorb) and on pure PLLA. In this study, chondrocytes were isolated enzymatically from human septal cartilage, resuspended in agarose, and transferred into the polymer scaffolds to create mechanical stability and retain the chondrocyte-specific phenotype. The cell–polymer constructs were then kept in perfusion culture for 1 week prior to subcutaneous transplantation into thymusaplastic nude mice. Significant differences were found between both nonwoven structures concerning matrix synthesis and matrix quality as well as vascular ingrowth. The PGA/PLLA scaffolds, with a degradation time of approximately 3 weeks *in vitro*, showed no significant differences from normal human septal cartilage in the amount of collagen types I and II, 24 weeks after transplantation. Thin fibrous tissue layers containing blood vessels encapsulated the transplants. PLLA constructs, which did not show strong signs of degradation even 24 weeks after transplantation, contained remarkably smaller amounts of cartilage-specific matrix components. At the same time, there was vascular ingrowth even in central parts of the transplants. In conclusion, polymer scaffolds with a short degradation time are suitable materials for the development of cartilage matrix products, while longer stability seems to inhibit matrix synthesis. Thus, this study suggests that *in vitro* engineering of human cartilage can result in a cartilage-like tissue when appropriate nonwovens are used.

However, in spite of their wide use as scaffolds in tissue engineering, some authors believe that nonwoven fibrous matrices require modification of their microstructure to organize cells in three-dimensional space with spatially balanced proliferation and differentiation in order to promote functional tissue development. In addition, the potential lack of structural stability for *in vivo* use has motivated the development of fiber-bonding methods to modify these nonwoven meshes into interconnected fiber networks with different shapes.

For example, Mikos et al.[1,3,31,32,35,42] developed a method of producing interconnected fiber networks by a fiber-bonding technique that involves the casting of a PLLA solution over a nonwoven mesh of PGA fibers. Solvent evaporation results in a composite material that consists of nonbonded PGA fibers embedded in a PLLA matrix. Fiber bonding occurs during a posttreatment at a temperature above T_m of PGA. Finally, the PLLA matrix is selectively dissolved in a nonsolvent for PGA, and a network of bonded PGA fibers is released. However, stipulations concerning the choice of the solvent, immiscibility of the two polymers, and their relative melting temperatures restrict the general application of the technique to other polymers.[1,3,31] In addition, this method of fiber bonding does not address the problem of creating scaffolds with complex three-dimensional shapes, but it has proven successful for producing hollow tubes that have been proposed for use in intestine regeneration.[1,3,31]

A different method, developed by Li et al.,[43] is based on the thermal compression of nonwoven polyethylene terephthalate (PET). Key parameters of the method described in this study, namely the temperature, pressure, and compression duration, were evaluated. The permanent deformation was obtained at elevated temperature under pressure and the viscoelastic compressional behaviors were observed, characterized by a distinct apparent modulus change in glass transition temperature region. A liquid extrusion method was further employed to analyze both pore size and its distribution for matrices with a porosity ranging from 84 to 93%. It was also found that a more uniformly distributed pore size resulted from thermal compression, and the isotropic nature of nonwoven fabrics was preserved because of the proportional reduction of the pore by compression. The thermally compressed fabric matrices with two different pore sizes (15 and 20 μm in pore radius) were used to culture human trophoblast ED27 and NIH 3T3 cells. It was found that cells cultured in the different pore-size PET matrices had different cell spatial organization and proliferation rates. The smaller pores in the matrix allowed cells to spread more readily and proliferate faster, while cells in the larger pores tended to form large aggregates and had lower proliferation rate. According to the authors, this thermal compression technique can also be applied to other synthetic fibrous matrices, including biodegradable polymers used in tissue engineering, to modify the microstructure according to their viscoelastic properties.

Despite the fact that a lot of work on fiber meshes has been focused on the use of synthetic polymers like the aforementioned PGA,[13,14,33,36–41] PLA,[41] and PET,[43] some natural-origin polymers, such as hyaluronic acid,[44,45] collagen,[46,47] and blends of starch with polycaprolactone,[9,48] have also been employed in the fabrication of fiber meshes for tissue engineering applications.

For example, a nonwoven mesh based on the benzyl ester of hyaluronic acid (HYAFF 11) was investigated for application as a scaffold material for the culture of human nasoseptal chondrocytes in tissue engineering procedures of cartilage reconstruction.[44] The fibers were produced by extrusion and had a diameter of about 20 μm under dry conditions. In aqueous solution, the material hydrates, which results in the swelling of the fibers to about twice their original diameter, and it undergoes spontaneous hydrolysis of the ester bonds. Due to the degradation process, the material becomes progressively hydrophilic and hydrated, reacquiring similarity to native HA polymer with time. Different techniques such as immunohistochemistry, scanning electron microscopy, and confocal laser scanning microscopy were used to study the behavior, morphology, and phenotype expression of the chondrocytes, which were initially expanded and then seeded onto these scaffolds. The nonwoven cell carrier allowed good viability and adhesion of the cells without any surface treatment with additional substances. Furthermore, the cultured cells expressed cartilage-specific collagen type II, indicating that they were able to dedifferentiate within the scaffold of HYAFF 11 and were

able to retain a chondrocyte phenotype even after a long period of *in vitro* conditions. Nevertheless, the expression of collagen type I, which was produced by dedifferentiated or incompletely dedifferentiated chondrocytes, was noticeable. Additional data were obtained by subcutaneous implantation of samples seeded with human cells in the *in vivo* model of the athymic nude mouse. The results after 1 month revealed the development of tissue similar to hyaline cartilage.[44]

A study by Gentlemann et al.[46] has assessed properties of collagen as a scaffolding biomaterial for ligament replacements. Mechanical properties of extruded bovine Achilles tendon collagen fibers were significantly affected by fiber diameter, with smaller fibers displaying higher tangent moduli and peak stresses. Mechanical properties of 125-μm-diameter extruded fibers (tangent modulus of 359.6 ± 28.4 MPa; peak stress of 36.0 ± 5.4 MPa) were similar to properties reported for human ligaments. Scaffolds of extruded fibers did not exhibit viscoelastic creep properties similar to natural ligaments. Collagen fibers from rat tail tendon (a well-studied comparison material) displayed characteristic strain-softening behavior, and scaffolds of rat tail fibers demonstrated a nonintuitive relationship between tangent modulus and specimen length. Composite scaffolds (extruded collagen fibers cast within a gel of type I rat tail tendon collagen) were maintained with and without fibroblasts under standard culture conditions for 25 days. Cell-incorporated scaffolds displayed significantly higher tangent moduli and peak stresses than those without cells. Because tissue-engineered products must possess appropriate mechanical as well as biological/chemical properties, data from this study should help enable the development of improved tissue analogues.

In other studies,[9,48] fiber meshes based on SPCL (a blend of starch with polycaprolactone, 30/70 wt%) were obtained by a fiber-bonding process, in this case consisting in the spinning, cutting, and sintering of the fibers with diameters of about 180 μm. The SPCL scaffolds obtained by this method have highly interconnected pores (see Figure 5.1) and exhibit a high potential for the regeneration of bone.

In one of these studies,[9] the aim was to investigate the effect of culturing conditions (static and flow perfusion) on the proliferation and osteogenic differentiation of rat bone marrow stromal cells seeded on two novel scaffolds exhibiting distinct porous structures. Specifically, scaffolds based on SEVA-C (a blend of starch with ethylene vinyl alcohol) and SPCL (a blend of starch with polycaprolactone) were examined in static and flow perfusion culture. SEVA-C scaffolds were formed using an extrusion process, while SPCL scaffolds were obtained by a fiber-bonding process. For this purpose, these scaffolds were seeded with marrow stromal cells harvested from femoras and tibias of Wistar rats and cultured in a flow perfusion bioreactor and in six-well plates for up

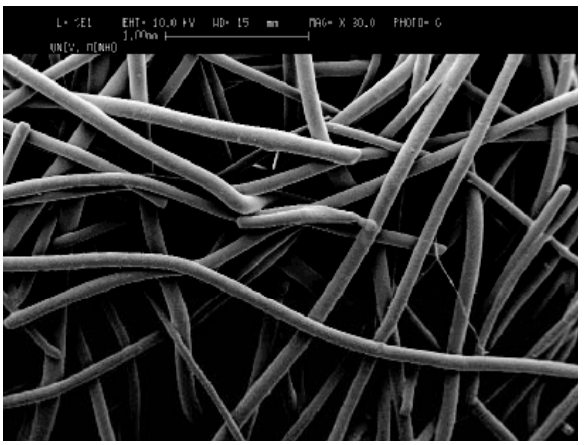

FIGURE 5.1 Typical structure of an SPCL (starch with polycaprolactone) fiber mesh obtained by a fiber-bonding process.

to 15 days. The proliferation and alkaline phosphatase activity patterns were similar for both types of scaffolds and for both culture conditions. However, calcium content analysis revealed a significant enhancement of calcium deposition on both scaffold types cultured under flow perfusion. This observation was confirmed by von Kossa-stained sections and tetracycline fluorescence. Histological analysis and confocal images of the cultured scaffolds showed a much better distribution of cells within the SPCL scaffolds than within the SEVA-C scaffolds, which had limited pore interconnectivity, under flow perfusion conditions. In the scaffolds cultured under static conditions, only a surface layer of cells was observed. These results suggest that flow perfusion culture enhances the osteogenic differentiation of marrow stromal cells and improves their distribution in three-dimensional starch-based scaffolds. They also indicate that scaffold architecture and especially pore interconnectivity affect the homogeneity of the formed tissue, making the fiber mesh structures more advantageous for tissue engineering applications, particularly those involving the use of flow perfusion bioreactors.

5.3.2 Electrospinning

Electrospinning is an attractive method for the production of fiber meshes for tissue engineering, particularly for the ability to generate fiber with very small diameters (ranging from several microns down to 100 nm or less) which mimic the nanometer scales of the fibers that compose the extracellular matrix of native tissues.[49]

The electrospinning technology is well suited to process natural and synthetic bioabsorbable polymers for biomedical applications,[50] such as poly(glycolic acid),[51] poly(lactic acid),[52] poly(*D,L*-lactide-co-glycolide),[53] polycaprolactone,[54] poly(lactic acid) and polycaprolactone-based blends,[55] collagen,[56–58] collagen–PEO blends,[59] and elastin[55] poly(ethylene-co-vinyl alcohol).[49] Some of these examples are further described in subsequent paragraphs.

In electrospinning, a polymeric solution or melt is injected with an electrical potential to create a charge imbalance and placed in proximity to a grounded target. At a critical voltage, a charge imbalance begins to overcome the surface tension of the polymer source, forming an electrically charged jet. The jet within the electrical field is directed toward the grounded target, during which time the solvent evaporates and fibers are formed. Electrospinning produces a single continuous filament that collects on the grounded target as a nonwoven fabric.[56]

Collagen is the natural scaffolding found in all tissues, and it has been explored extensively for use as a tissue engineering scaffold with limited success. The electrospinning of collagen type II and subsequent chondrocyte seeding was investigated for potential use in cartilage tissue engineering.[57] The electrospinning process utilized lyophilized chicken sternal cartilage collagen type II suspended in 1,1,1,3,3,3-hexafluoro-2-propanol and demonstrated that collagen type II could be electrospun to form nonwoven fibrous mats composed of type II fibers that ranged from 110 nm to 1.8 μm in diameter. The fiber diameter was dependent on the type II concentration in solution, with a higher concentration producing the larger diameters. This preliminary chondrocyte seeding study demonstrated that electrospun collagen type II scaffolds support cell growth and are readily infiltrated. In conclusion, the feasibility of collagen type II electrospinning has been demonstrated, and the novel scaffolds produced are composed of nano- to micron-scale fiber diameters that have been shown to be compatible with chondrocytes.[57]

In a different study, type I collagen–PEO fibers and nonwoven fiber networks were produced by the electrospinning of a weak acid solution of purified collagen at ambient temperature and pressure.[60] As determined by high-resolution SEM and TEM, fiber morphology was influenced by solution viscosity, conductivity, and flow rate. Uniform fibers with a diameter range of 100 to 150 nm were produced from a 2-wt% solution of collagen–PEO at a flow rate of 100 ml/min. Ultimate tensile strength and elastic modulus of the resulting nonwoven fabrics was dependent on the chosen weight ratio of the collagen–PEO blend. H-1 NMR dipolar magnetization transfer analysis suggested that the superior mechanical properties, observed for collagen–PEO blends of weight ratio

1:1, were due to the maximization of intermolecular interactions between the PEO and collagen components. The process previously outlined provides a convenient, nontoxic, nondenaturing approach for the generation of collagen-containing nanofibers and nonwoven fabrics that have potential application in wound healing, in tissue engineering, and as hemostatic agents.

A study by Yoshimoto et al.[54] assessed the potential of electrospinning as an alternative scaffold fabrication technique to engineer bone *in vitro* using a rotational oxygen-permeable bioreactor. For this purpose, microporous, nonwoven poly(ε-caprolactone) (PCL) scaffolds were made by electrostatic fiber spinning and seeded with mesenchymal stem cells (MSCs) derived from the bone marrow of neonatal rats. The cell–polymer constructs were cultured with osteogenic supplements under dynamic culture conditions for up to 4 weeks. The cell–polymer constructs maintained the size and shape of the original scaffolds. Scanning electron microscopy (SEM) and histological and immunohistochemical examinations showed the penetration of cells and abundant extracellular matrix in the cell–polymer constructs after 1 week. SEM showed that the surfaces of the cell–polymer constructs were covered with cell multilayers at 4 weeks. In addition, mineralization and type I collagen were observed at 4 weeks. This suggests that electrospun PCL is a potential candidate scaffold for bone tissue engineering.[54]

Other researchers[50,61] have investigated the potential of electrospun scaffolds to function also as drug delivery devices, based on the possibility of incorporating therapeutic compounds into solutions for electrospinning. In one of these studies, the release of tetracycline hydrochloride (used as a model drug) from electrospun poly(ethylene-co-vinyl acetate), poly(lactic acid), and a blend of these two polymers was assessed.[61] In another work, researchers studied the release of the antibiotic drug Mefoxin from a poly(*D,L*-lactic acid) (PDLA) fiber membrane obtained by electrospinning.[50] In general, these studies demonstrate that the simplicity of this process and the wide range of polymers that can be used may enable a broader application of electrospun polymer matrices in controlled release.[61] Furthermore, the unique morphology of electrospun meshes, in particular their large surface-to-volume ratio and short diffusion passage length (and also consequently degradation properties), may provide several advantages for the controlled release of drugs, proteins, or even cells over bulk films or additional scaffold architectures.[50]

5.4 PARTICLE AGGREGATION

In the past few years, there has been a trend toward the development of increasingly sophisticated scaffold materials that may enable the combination of several functions within the same device.[62–65] This is a very attractive characteristic for tissue engineering scaffolds, as these materials are required to perform a very complex role in the development of tissue substitutes.

One innovative approach to designing polymer-based scaffolds is based in the microsphere technology. The so-called aggregation of polymer microparticles method consists in the aggregation, by physical or chemical means, of microparticles.[35] The porosity obtained in this type of scaffold can be easily controlled by the microsphere diameter that will create the interstices when the particles are aggregate. If an increased pore size is desired, it is also possible to use macroparticles, as our group has done.[66] Typically, the function of the tissue-engineered scaffolds is to create and maintain a space and to provide a support for cell adhesion. However, these scaffolds can also serve as carriers for the delivery of bioactive agents such as growth factors in order to manipulate cellular processes within the scaffold microenvironment as well as in the surrounding implantation site. This is clearly one main advantage of this processing method, allowing for the controlled release of earlier encapsulated growth factors or cells from the microspheres.

Only a few works using this method can be found in the literature.[21,67,68] Laurencin and his coworkers[21] have been developing gel and sintered microsphere (incorporating hydroxylapatite) matrices based in poly(lactide-co-glycolide) microspheres. Both microsphere matrices are designed using the random packing of PLAGA microspheres to create a three-dimensional porous structure. The effects of matrix composition and processing (such as stirring and heating time) were studied

in order to optimize the process, and it was shown that both parameters affected the properties of the scaffolds, making it possible to obtain a range of pore sizes, volume, and mechanical properties that seem suitable for cancellous bone tissue engineering applications.[21] The values for the pore size were found to be in the 60 to 150 μm range that the authors find interesting for the potential use of the implant as a negative template for cancellous bone replacement, meaning that the pore size of the implant matches that of the cancellous bone struts.[21] Concerning the mechanical properties, the modulus is in the 350 to 240 MPa range, depending on the microsphere diameter for the sintered matrices. The gel microsphere matrices present the highest modulus range (630 to 1650 MPa), depending on the HA concentration and the stirring time.[21] The *in vitro* osteoconductivity and degradation profiles were also studied.[20] Osteoblasts and fibroblasts were seeded in the scaffolds, and cross-sectional images indicated that cellular proliferation had penetrated into the matrix approximately 700 μm from the surface of the sintered microsphere matrices.[20] The cells migrated through the matrix using cytoplasmatic extensions to bridge the structure, which can be a disadvantage due to the pore covering by the cells, which is an ongoing discussion. Regarding the degradation behavior, it was found that it is dependent on the molecular weight, copolymer ratio, and pore volume.[20] From this data, the authors established an optimal degradation behavior that can be controlled by changing the above parameters. Furthermore, in another study,[69] the same authors establish the optimal structural parameters for the biomimetic structure with basis in the different osteoconductive scaffolds, based on the synergistic balance between porosity and mechanical properties. In the referred works,[20,69] no data on bioactive agents were presented.

On the other hand, Shea and his coworkers[67] have used this potential technique to produce scaffolds for tissue engineering, loading previously the material with plasmid DNA. The work compares mainly the DNA release profiles obtained through different shapes (compressed discs, nonporous foamed discs, and porous scaffolds). It was concluded that the fusion of the drug-loaded microspheres into a porous scaffold via a gas-foaming technique efficiently retains the DNA within the scaffold and produces a sustained release up to 21 days that differs from the one observed for the individual microspheres (24 hours). In a later study,[70] the authors have optimized the microsphere preparation method using a cryogenic double-emulsion method in order to maximize the DNA incorporation efficiency.

In a study by Botchwey et al.,[68] a novel approach was utilized in order to grow *in vitro* mineralized bone tissue using lighter-than-water polymeric scaffolds in a high-aspect-ratio rotating bioreactor. This approach was adapted from polymer microencapsulation methods for the formation of hollow, lighter-than-water microcarriers of degradable poly(lactic-co-glycolic acid). Scaffolds were fabricated by sintering together lighter-than-water microcarriers from 500 to 860 microns in diameter to create a fully interconnected, three-dimensional network with an average pore size of 187 microns and aggregate density of 0.65 g/ml. Motion in the rotating bioreactor was characterized by numerical simulation and by direct measurement using an *in situ* particle-tracking system. Scaffold constructs established a near-circular trajectory in the fluid medium with a terminal velocity of 98 mm/s while avoiding collision with the bioreactor wall.[68] Preliminary cell culture studies on these scaffolds show that osteoblast-like cells readily attached to microcarrier scaffolds using controlled seeding conditions with an average cell density of 6.5×10^4 cells/cm^2. The maximum shear stress imparted to attached cells was estimated to be 3.9 dynes/cm^2. In addition, cells cultured *in vitro* on these lighter-than-water scaffolds retained their osteoblastic phenotype and showed significant increases in alkaline phosphatase expression and alizarin red staining by day 7, compared with statically cultured controls.

Nevertheless, this method has some disadvantages. In fact, one of its main drawbacks is that the porosity generated by using microspheres is very low; as mentioned before, the size of the interstices between the aggregated particles is directly related to the particle diameter. As stated earlier, if an increased pore size is desired, it is also possible to use macroparticles, as demonstrated in a study on the development of scaffolds based on the aggregation of chitosan-based macroparticles.[66] These scaffolds exhibit very promising mechanical properties (compressive modulus of

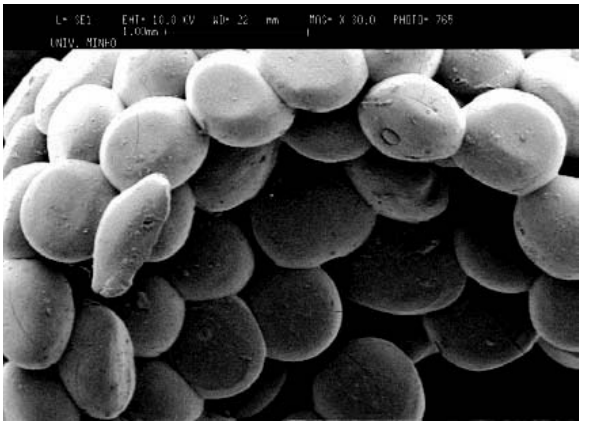

FIGURE 5.2 Typical morphology obtained for the scaffolds produced by chitosan-based particle aggregation (magnification 30×).

around 300 MPa, depending on the matrix processing). These values can be further increased up to 400 MPa with the incorporation of a bioactive reinforcement phase (in this study, hydroxylapatite was used). The porosity was found to vary between 200 and 600 μm, depending on the heating temperature and time. Furthermore, the particle size can be controlled by reaction parameters that will allow for the production of scaffolds with a wide range of pore sizes. One main advantage of this method is the possibility of controlling the water uptake ability by crosslinking the chitosan polymer; this will decrease the hydration degree significantly and will obviously influence the drug release, allowing for a wide range of release profiles, depending on the required application. Figure 5.2 shows the typical morphology obtained for the developed scaffolds.

5.5 CONCLUSIONS

Much of the discussion within the present chapter has focused on the challenge of producing adequate three-dimensional multifunctional constructs from biodegradable polymers, as these materials present an outstanding potential for providing the necessary support (from the materials science point of view) to new and developing tissue engineering strategies.

In the authors' view, it is clear that the processing technologies that were described in detail present an outstanding potential for providing adequate scaffold structures to be used in a new generation of tissue engineering strategies.

However, from the materials science point of view, the research on new and improved scaffold devices for bone tissue engineering should continue to challenge tissue engineers to come up with new polymers and new processing methodologies that can help to enhance existing medical therapies to treat hard-tissue defects and diseases that affect millions of people all over the world.

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6 Design and Fabrication of Scaffolds via Solid Free-Form Fabrication

Dietmar W. Hutmacher

CONTENTS

6.1 INTRODUCTION

Scaffold-based tissue engineering concepts involve the use of combinations of viable cells, biomolecules, and a structural matrix combined into a "construct" to promote the repair and regeneration of tissues. The scaffold construct is intended to support cell migration, growth, and differentiation and guide tissue development and organization into a mature, healthy state. In parallel or delayed, the scaffold component undergoes degradation or resorption via production of biocompatible, excretable, or metabolizable by-products.[1,2]

The field is still young and many different approaches are under experimental investigation. It is by no means clear what defines ideal scaffold–cell or scaffold–neotissue constructs, even for a specific tissue type. The considerations are complex and include architecture, structural mechanics, surface properties, degradation products, and composition of biological components, along with the changes in all of these factors with time.

Successful tissue engineering constructs will have certain minimum requirements for biochemical as well as chemical and physical properties. Scaffolds must provide sufficient initial mechanical strength and stiffness to substitute for the mechanical function of the diseased or damaged tissue that it aims to repair or regenerate. Scaffolds may not necessarily be required to provide complete mechanical equivalence to healthy tissue, but stiffness and strength should be sufficient to at least support and transmit forces in a healing site — e.g., the wound contraction forces in the case of skin tissue engineering, or on patient activity in early-stage recovery in the case of cartilage engineering, and callus formation in bone engineering.

Cell and tissue remodeling is important for achieving stable biomechanical conditions and vascularization at the host site. Hence, the 3-D scaffold–tissue construct should maintain sufficient structural integrity during the *in vitro* or *in vivo* growth and remodeling process. The degree of remodeling depends on the tissue itself (e.g., skin 4 to 6 weeks, bone 4 to 6 months) and its host anatomy and physiology.[3]

Scaffold architecture has to allow for initial cell attachment and subsequent migration into and throughout the matrix, mass transfer of nutrients and metabolites, and provision of sufficient space for development and later remodeling of organized tissue. The porosity and internal space within a degradable scaffold will increase with time, allowing increased space for tissue development or remodeling. The degradation profile might need to be synchronized based on two overall strategies described and discussed in the next sections.

In addition to these essentials of mechanics and geometry, a suitable construct will possess surface properties that are optimal for the attachment and migration of cell types of interest in an applicable three-dimensional structure. The external size and shape of the construct must also be considered, especially if the construct is customized for an individual patient.

In addition to considerations of scaffold performance based on a holistic tissue engineering strategy, practical manufacturing considerations arise. For realistic and practical clinical applications, it must be possible to manufacture in a reproducible, controlled fashion at an economic cost and speed. The manufacturing process might allow the presence of biological components; for example, it may be necessary to allow cell seeding or incorporation of biomolecules that may be soluble or heat labile. Tissue engineering constructs by reason of their very complexity, particularly over small length scales, have stimulated the exploration of innovative and special manufacturing techniques.

In this chapter, we try to define the needs versus the wants of tissue-engineered scaffold/cell/ECM constructs, with particular emphasis on the matrix properties in general, and attempts to define some broad constraints for the properties of suitable materials and morphology with an emphasis on bone tissue engineering. We then review the current state of the art in manufacturing techniques applied to scaffold fabrication in the context of these constraints on scaffold design.

6.2 SCAFFOLD DESIGN AND PROPERTIES

6.2.1 Morphology/Architecture

Tissues are organized into three-dimensional (3-D) structures as functional organs and organ systems. Each has its specific characteristic architecture according to its biological function. The architecture provides the requisite mechanical and structural integrity and appropriate channels for mass transport and spatial cellular organization. Mass transport includes signaling molecules, nutritional supplies, and metabolic waste removal. Spatial cellular organization determines cell–cell and cell–matrix interactions and is critical to the normal tissue and organ function. Native extra-

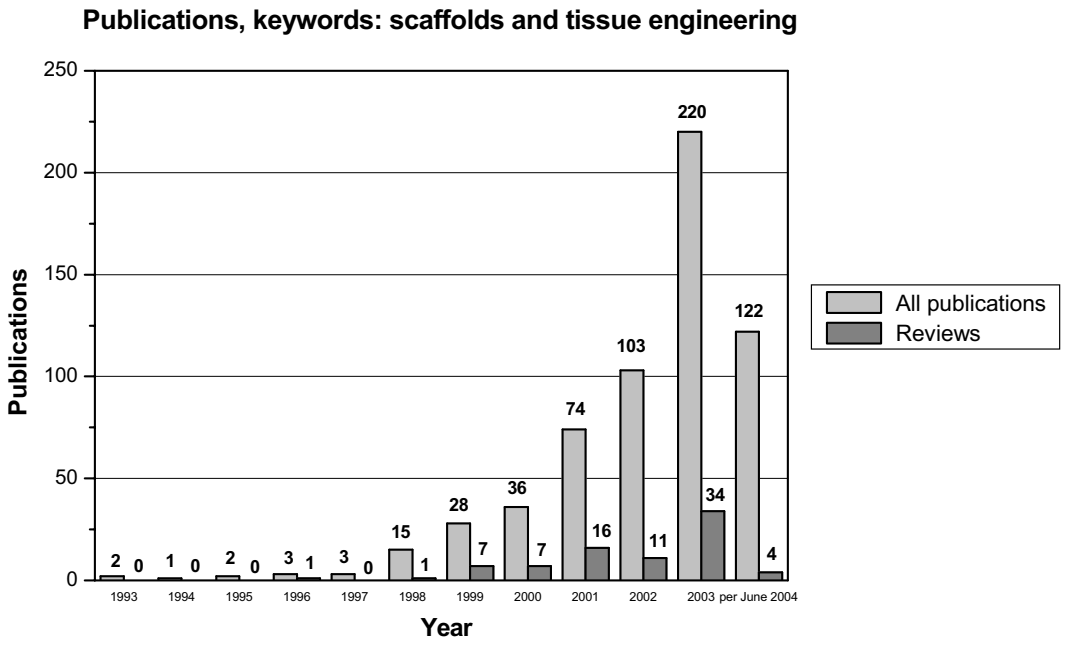


FIGURE 6.1 Number of research papers on tissue engineering and scaffold. The increase observed from 1992 to 2003 reveals heightened interest in the design and fabrication of suitable scaffolds. Published/recorded in PubMed from 1993–2004.

cellular matrices (ECMs), in combination with structural connective tissue elements, form the natural structural scaffolding for three-dimensional organization of cells in a tissue and also provide the cells with a structural and functional network to regulate their cell- and tissue-specific protein and gene expression.[4]

Tissue engineering constructs generally employ natural or synthetic materials engineered into three-dimensional scaffolds to grow soft and hard tissues by using isolated cells. To engineer a tissue or organ with a specific function, scaffold design and material play a critical role in allowing for the appropriate cell distribution and in guiding the tissue regeneration in three dimensions. Therefore, to develop a scaffold for tissue engineering, the architectural design concerning the spatial cellular distribution (initially via cell adhesion and subsequently by cell migration, proliferation, and differentiation), mass transport conditions, and tissue function is very important.[5]

6.2.1.1 Physical Properties of Scaffolds

The strong increase in interest to design and fabricate suitable scaffolds is reflected in the statistics shown in Figure 6.1.

Today, scaffolds might be categorized from a physical point of view into three groups, namely cellular solids, textiles, and injectable systems. Ashby and Gibson[6] classified porous solids into two general groups: foams (Figure 6.2) and honeycombs (Figure 6.3). A honeycomb consisted of a regular two-dimensional array of polygonal pores, each defined by a wall shared between adjacent pores. The pores were packed in planar arrays like the hexagonal cells of a honeycomb.

The ASTM terminology[7] for porous materials is similarly classified into three groups: interconnecting (open pores), nonconnecting (closed pores), or a combination of both. When the pores were open, the foam material was usually drawn into struts, forming the pore edges. A network of struts produced a low-density solid with pores connecting to each other through open faces. When the pores were closed, a network of interconnected plates produced a higher-density solid. The closed pores were sealed off from adjacent neighbors.

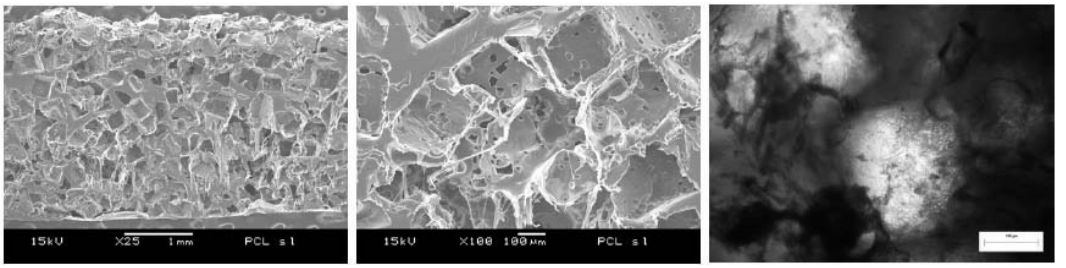


FIGURE 6.2 The factors governing scaffold properties are complex and include considerations about architecture, mechanics, surface properties, degradation products, and composition of biological components and the changes in these factors with time. Usually, tissue engineering scaffolds have certain minimum requirements for biochemical and physical properties. Scaffolds must provide sufficient initial mechanical strength and stiffness to substitute for the mechanical function of the diseased or damaged tissue. Moreover, scaffold architecture has to allow initial cell attachment and subsequent migration into the matrix, mass transfer of metabolites, and sufficient space for development of a vasculature system and remodelling of organized tissue matrix. Scaffolds made by so-called conventional fabrication technologies (Hutmacher 2000) can be ordered according to stochastic, fractal, or periodic principles. However, most of the scaffold morphologies produced by that method show poor performance due to the fact that their matrix architectures do not allow the cells to migrate into the inside of the scaffolds. The scaffold above was produced by the solvent casting and particle leaching method described in the literature reviewed by Agrawal et al. (1997). Seeding and culturing did show the effect reported above and by many other groups in original papers.

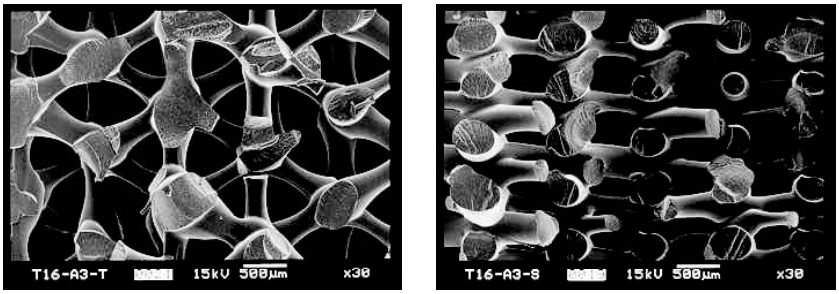


FIGURE 6.3 Freeze-fractured surface of PCL scaffold with 0/60/120º lay-down pattern and 70% porosity, fabricated via fused deposition modelling (T16 tip, RW ~ 0.406 mm, FG ~ 0.711 mm, ST ~ 0.254 mm). Scaffold morphology observed under SEM, (left) plan view showing honeycomb pattern with triangular pores, (right) side view showing channel architecture and 100% interconnectivity.

Ashby and Gibson[6] found that the mechanical properties of a porous solid depended mainly on its relative density, the properties of the material that made up the pore edges or walls, and the anisotropic nature, if any, of the solid. In general, the stiffness E^* and yield strength σ^* in compression of porous solids were each related to the relative density by a power law relationship. Given that most constructs will require a high degree of porosity to accommodate mass transfer and tissue development, the volume fraction of the scaffold should be necessarily low. In all but the most biomechanically challenging applications, it is likely that the test will be to achieve sufficient stiffness and strength in a highly porous structure to provide adequate mechanical integrity. One of the most demanding applications will be the repair and generation of musculoskeletal tissues, particularly bone, where scaffolds need to have a high elastic modulus and compression strength in order to provide temporary mechanical support without showing symptoms of fatigue or failure, to be retained in the space they were designated for, and to provide the tissue with adequate space for growth. One of the fundamental challenges of scaffold design and materials selection is that, to achieve sufficient strength and stiffness, the scaffold material

must have sufficiently high interatomic and intermolecular bonding but at the same time needs to have a physical and chemical structure that allows for hydrolytic attack and breakdown.

6.2.1.2 Pore Morphology and Overall Scaffold Architecture

Based on studies of the mechanical properties and function of the cytoskeleton,[8,9] a group[10] proposes to design scaffolds based on a concept that they named "tensegrity." Geodesic 3-D constructs were designed by applying tensegrity so that the entire scaffold structure evenly distributes and balances mechanical stresses. The walls, layers, or struts that make up the interconnecting scaffold framework are connected into triangles, pentagons, or hexagons, and the structure is designed so that all members bear approximately equal stress. The mechanical rationale of the tensegrity design concept is essentially a restatement of straightforward engineering design principles in which structures are designed in such a way as to make the most efficient use of materials by distributing loads evenly without placing undue stress on any individual component. This principle has been understood for centuries in the area of civil engineering. Gothic architects used elaborate skeletal structures of stone columns and ribs supported by arches and buttresses to construct large buildings and structures such as churches, bridges, etc. A mathematical description of the optimization of space frames was first proposed by Maxwell[11] and is known as Maxwell's lemma. A detailed discussion of the mathematical principles involved is given by Parkes and Wainwright et al.,[12,13] who considered the application of these principles to biological structures and pointed out that in most cases, the ideal structures — i.e., those that maximize stiffness and minimize volume of material — are in the form of orthogonal nets. Fortunately, such structures are easily manufactured via solid free-form fabrication (SFF).

In the human body, the development and remodeling of skeletal structures make the most efficient use of material and design properties, and as a result, nature builds tissues where stresses are evenly distributed. From this principle, it would be expected that for a given material (e.g., cortical and cancellous bone), structures would be designed in such a way as to produce approximately equivalent stresses within the strength of the material. Biewener[14] showed that this was the case for limb bones, with stresses during normal locomotion being very similar in the limb bones of a range of species across length scales from mouse to elephant. More elaborate examples are seen in the trabecular structure of the human hip, spine, and knee joint, where the struts of cancellous bone are aligned with the principle stress directions to make most efficient use of the different structural members within a design concept. Hence, it is a challenge for the material scientist and engineers in a tissue engineering team to design and build scaffolds based on those principles.

6.2.1.3 Nutrient Supply and Vascularization *In Vivo*

A constraint to the size of a tissue engineering construct arises from the limitations of diffusion of nutrients and metabolites through the scaffold pores. In normal tissues, there is a diffusion limit of approximately 100 to 200 μm,[15] which is the maximum distance between any cell and the nearest blood capillary, beyond which there is inadequate supply of oxygen and nutrients. An interconnected macropore structure — with pores bigger than 300 μm — enhances the diffusion rates to and from the center of a scaffold; transportation of the nutrients and by-products is not sufficient for large scaffold volumes, and the same diffusion-based limits apply. For tissue engineering, the creation of a vascularized bed ensures the survival and function of seeded cells, which have access to the vascular system for nutrition, gas exchange, and elimination of metabolites. The vascularization of a scaffold may be compromised by purely relying on capillary ingrowth into the interconnecting pore network from the host tissue. Therefore, the time frame has to be considered for the capillary system to distribute through larger scaffold volume. It may also be possible to control the degree and rate of vascularization by incorporating angiogenic factors in the degrading matrix of the scaffold.

In order to overcome the short-term requirements for nutrient supply to the inner regions of bulk constructs, Hollister and coworkers[16] have suggested the use of microsurgical techniques to place blood vessels into the construct or the use of "global pores" — i.e., continuous channels penetrating through the construct — to provide conduits for flow of nutrients and metabolites. Hutmacher's group[17,18] did show that scaffolds of such a design with large honeycomb-like pores and a fully interconnected channel-like gross morphology allow *in vivo* rapid vascularization of the *in vitro* engineered scaffold–neotissue construct.

6.2.1.4 *In Vitro* Culture

Considerations of diffusion and hydrodynamics also apply in *in vitro* culture of tissue engineering constructs.[19] The growth of neotissue (scaffold/cell/ECM construct)[20] is dependent on the immediate environment that the constructs are subjected to. The factors that can modulate growth include temperature, culture medium, chemical factors, and mechanical environment, including fluid flow and perfusion. Each of these individually can have a dramatic impact on the growth of the tissue, although if controlled, they can be used as major positive modulators.

Providing the three-dimensional scaffold–cell constructs with nutrients may rely on passive diffusion, or nutrients may be more actively delivered by direct perfusion. Tissues that have been manufactured to date have relied on diffusion, although tissues envisioned for future products will require a more active delivery process. In a number of long-term tissue engineering studies, it has proven extremely difficult in a static environment to promote the high-density three-dimensional *in vitro* growth of human tissues that are deprived of their normal vascular sources of nutrients and gas exchange.[19]

The culture dish/flask can be defined as the simplest and most widely used bioreactor today. This product provides an environment that is sterile, easy to use, simple, and economical to manufacture. However, the fact that the culture dish offers only a static 2-D environment and requires individual manual handling for medium exchange, cell seeding, etc., ultimately limits its usefulness when large numbers are required. Scale-up of the culture dish, such as the addition of multiwell plates, only goes so far.

Cells in monolayer culture are not generally nutrient-limited. This is because passive diffusion is more than adequate to supply a 10-micron-thick cell layer. In contrast, the supply of oxygen and soluble nutrients becomes critically limiting for the culture of tissues that are thicker than 100 to 200 microns. This diffusion limitation is partly alleviated by stirring of the culture medium. Continuous flow in a roller bottle or spinner flask (Figure 6.4) provides continuous exposure of the tissue surface to fresh nutrients as well as to (controllable) shear forces.

One means of overcoming some of these constraints is by the use of bioreactors that continuously supply physiological nutrients and gases. Computer control of bioreactors (Figure 6.5) can be used to regulate the required cell/tissue culture conditions for a long period of time. A bioreactor can provide complex control that extends beyond simple considerations of mass transfer to control of hydrodynamic and biochemical factors in the cell environment. For example, an appropriately designed bioreactor can both enhance mass transfer and provide hydrodynamic stimulation at the tissue–fluid interface, e.g., by fluctuations in fluid velocity and stresses.[19]

Special and custom-made bioreactor design can provide sometimes-complex mechanical, electrical, and fluid-flow-derived cues to cultivated tissues. Mechanical stimuli are not the only physiologically and developmentally relevant physical inputs that may be harnessed in bioreactors. Wainwright et al. pointed out that natural selection has driven biological processes to develop efficient solutions to mechanical design within the constraints of the materials available. These solutions are achieved by adaptive remodeling responses to simple stimuli of force, pressure, and chemical gradients. What is most remarkable is the very plasticity of the biological remodeling processes. In considering the requirements for ideal tissue engineering constructs, it is unnecessary, indeed probably fruitless, to attempt to provide a precise, highly detailed guide to regenerating

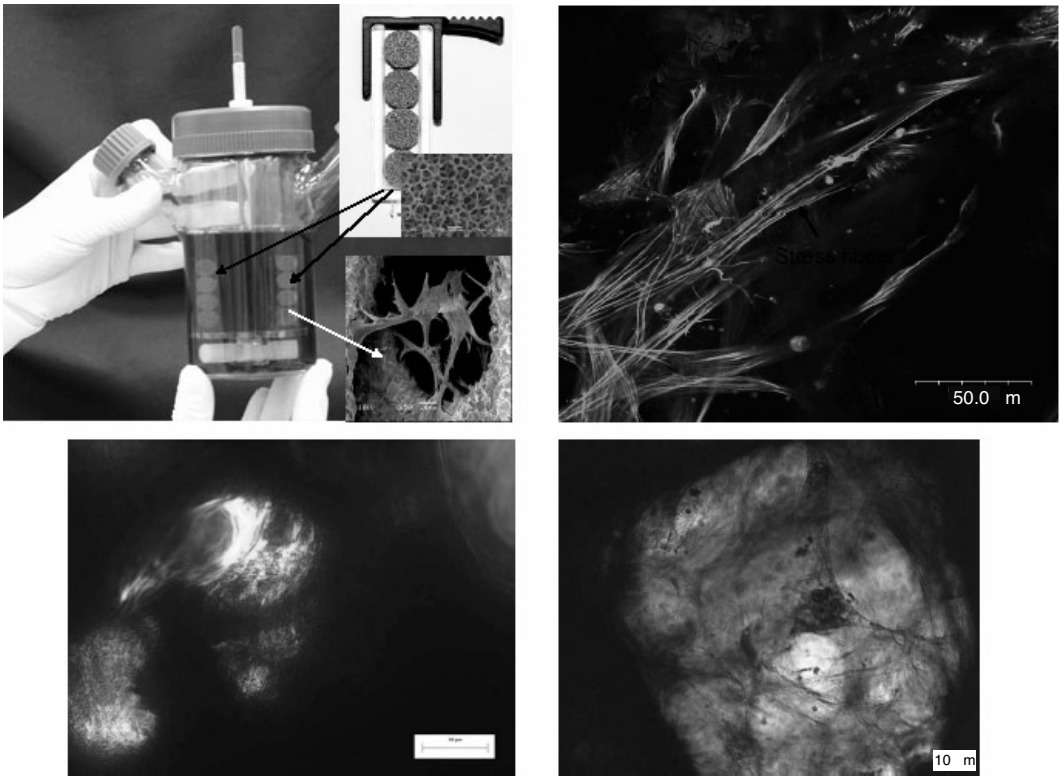


FIGURE 6.4 To ensure a sufficient number of tissue-specific cells without donor site morbidity, efficient *in vitro* culture methods to rapidly expand MSCs to high cell density while retaining their pluripotency, particularly over extended culture periods, are of practical importance to both clinical and research efforts involving these cells. Culture optimization protocols must thus be designed to specifically target specific cell populations. Selection or enrichment processes can be used to start a homogeneous cell population to enhance the frequency of stem cells. Optimization of culture conditions for stem cell growth and differentiation has been further hindered by the need for retrospective assays of cell function to quantify stem cell responses to candidate culture parameters. The need for improved and user-friendly cultivation methods is driven by the fact that many potential therapeutic applications are limited by the availability of stem cells or precursor cells. Based on this background, a group at the Harvard Medical School has developed a cell expansion system (www.cytomatrix.com).

tissue; instead one should concentrate on the general conditions and principal stimuli that will guide the natural remodeling processes to production of normal, mature tissue.

6.2.2 Materials and Properties

The considerations of mechanical properties and degradation behavior that have been discussed for the scaffold structure also clearly apply to materials selection. In addition, the principle biological interactions (protein and peptide adhesion, cell adhesion, migration, proliferation, and differentiation) are primarily a function of the surface properties of the scaffold material. Consideration must be given to the requirements for general biological safety that apply to all implant materials. That is, the scaffold should be fabricated from a material that does not elicit adverse biological responses such as immunotoxicity or clinically detectable primary or secondary foreign body reaction.[20]

Several scaffold materials have been investigated for tissue engineering applications, including polymers of synthetic origin such as polyglycolide (PGA) and polylactide (PLA) and natural polymers such as collagen and chitin. In the 1990s, scaffold design and fabrication were directly

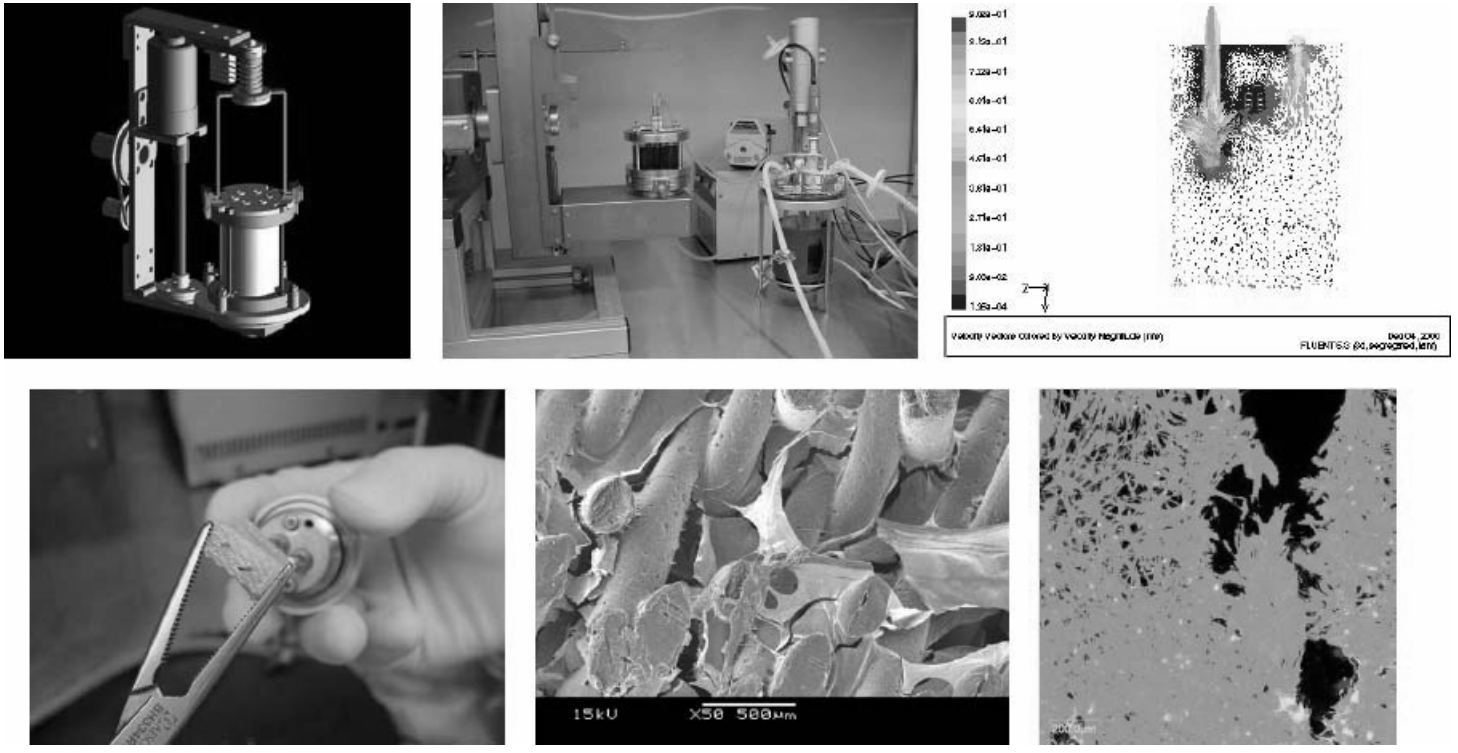


FIGURE 6.5 Over the past year, the National University of Singapore, in collaboration with the Singapore Polytechnic, has been developing a bioreactor for growing fragile cell cultures into complex, three-dimensional, mature facsimiles that mimic the structure and function of the parent tissue. These replacement tissues are being realized through the use of biodegradable polymer scaffolds; temporary/permanent substrates, which facilitate cell attachment, proliferation, retention, and differentiated tissue function. To optimize gas transfer and nutrient delivery, as well as to recreate the fluid dynamic environment present within the body, a dynamic system was chosen, which affords biaxial motion for the tissue culture vessel. Both axes of the reactor are independently computer controlled, allowing full or partial cyclic motion. For aseptic operation, the compact dimensions of the reactor allow it to be placed within a biohazard hood during set-up operation. Temperature control and gas transfer into the closed loop system are achieved through a membrane oxygenator, while the continuous flow of media and nutrients is maintained through the culture chamber by a peristaltic pump. Unlike conventional (perfused) bioreactor systems, the biaxial bioreactor has no impellers, airlifts, or agitators that could damage the fragile tissue. Computer modelling of the biaxial bioreactor has also revealed superior flow patterns free of damaging shear stresses, when compared to conventional bioreactors including single-axis designs. (From Hutmacher, D.W., *Biomaterials*, 21, 2529, 2000. With permission.)

based on commercially available bioresorbable implants and devices. More recent techniques were developed based on modern polymer processing and textile technologies. In the latter scaffold fabrication techniques, the viscous behavior of the polymers above their glass transition or melting temperatures and their solubility in various organic solvents were two important characteristics that dictated the type of fabrication process used. This work has been reviewed elsewhere.[21,22]

Currently, the design and fabrication of scaffolds in tissue engineering research is driven by three material categories:

1. Established biodegradable and bioresorbable polymers with existing clinical applications such as degradable sutures, including collagen, polyglycolide (PGA), polylactides (PLLA, PDLA), and polycaprolactone (PCL).
2. A number of other polymers without preexisting clinical applications, such as polyorthoester (POE) and polyanhydrides, polyfumarates, etc.
3. Specially tailored polymeric biomaterials, such as poly(lactic acid-co-lysine), which can selectively shepherd specific cell phenotypes and guide the differentiation and proliferation into the targeted functional premature or mature tissue.

Aliphatic polyesters, such as PLA, PGA, and PCL, are by far the most commonly used synthetic polymer materials for manufacture of scaffolds. In general, polymers of the poly(α-hydroxy acids) group undergo bulk degradation. The molecular weight of the polymer is measurably decreased on day 1 (PGA, PDLA) or after a few weeks (PLLA, PCL) upon placement in an aqueous media.[23] However, mass loss does not start until the molecular chains are reduced to a size that allows them to freely diffuse out of the polymer matrix.[24] This phenomenon, described and analyzed in detail by a number of research groups,[21,25,26] results in accelerated degradation and resorption kinetics until the physical integrity of the polymer matrix is compromised. The mass loss is accompanied by a release gradient of acidic by-products.

In vivo, massive release of acidic degradation and resorption by-products results in inflammatory reactions. If the capacity of the surrounding tissue to eliminate the by-products is low, due to poor vascularization or low metabolic activity, the chemical composition of the by-products may lead to local temporary disturbances. One example of this is the increase of osmotic pressure or pH manifested through local fluid accumulation or transient sinus formation from fiber-reinforced polyglycolide pins applied in orthopedic surgery.[26]

Potential problems of biocompatibility in tissue engineering, by applying degradable, erodable, and resorbable polymer scaffolds, may also be related to biodegradability and bioresorbability.[21] Therefore, it is important that the 3-D scaffold–cell construct be exposed at all times to sufficient quantities of neutral culture media, especially during the period where the mass loss of the polymer matrix occurs. Rotter et al.[27] report one experiment in which a large amount of degradation by-products led to the death of an entire group of scaffold–cell constructs.

For bone tissue engineering, the incorporation of a tricalcium phosphate (TCP) and hydroxyapatite (HA) — and for other tissues, basic salts — into a fast polymer matrix system such as PGA and PGA/PLA produces a hybrid/composite material that can buffer the acidic by-products. These inorganic fillers allow one to tailor the desired degradation and resorption kinetics of the polymer matrix. A composite material would also improve biocompatibility and hard tissue integration because ceramic particles, which are embedded in the polymer matrix, allow for increased initial flash spread of serum proteins compared with the more hydrophobic polymer surface. Bone tissue engineers were primarily responsible for building on the basic resorption products of HA or TCP that would buffer the acidic resorption by-products of the aliphatic polyester and may thereby help to avoid the formation of an unfavorable environment for the cells due to a decreased pH.[28,29]

In consideration of scaffold degradation and mechanical properties, different needs may apply, depending on the extent of preimplant *in vivo* culture phase. A number of alternate strategies have been used. In the simplest case, scaffolds may be implanted directly into tissues without any cell

seeding, drawing only on migration of available cells from the host site. A slightly more complex approach is to seed the scaffold with cells, possibly with an *in vivo* incubation of several hours to allow cell attachment, followed by immediate implantation into the host site. The most complex approach involves seeding followed by extended periods (up to several weeks) of *in vitro* culture to allow cellular differentiation and reorganization into some level of partially mature tissue (also defined as neotissue[17]), which is then implanted into the host site. This *in vitro* culture is likely to be conducted using bioreactors and stimulus of the cells with bioactive molecules (growth factors, etc.) to direct differentiation, proliferation, and organization.

Clearly, the time-dependent mechanical and degradation requirements will differ substantially, depending on the targeted tissue itself and the particular strategy adapted to either repair or regeneration. The considerations of mechanical requirements are perhaps most important in tissue engineering of load-bearing tissues such as bone and cartilage; however, in principle, any tissue undergoes certain types of biomechanical loading during wound healing and tissue remodeling.

In the case where a seeded scaffold is implanted with no or minimal *in vitro* incubation, the physical scaffold structure supports the polymer/cell/tissue construct from the time of cell seeding up to the point where the hard tissue transplant is remodeled by the host tissue.[20] In the case of load-bearing tissue such as articular cartilage and bone, the scaffold must also provide sufficient temporary mechanical support to withstand *in vivo* loading. In such cases, the material must be selected or designed with a degradation and resorption rate such that the strength of the scaffold is retained until the tissue-engineered transplant is fully remodeled by the host tissue and can assume its structural role.

Mechanical loading may directly affect the degradation behavior. Thomson et al.[30] studied a poly(*D,L*-lactide-co-glycolide) matrix under cyclic compressive loading. They concluded that changes in surface deformation and morphology show that compressive loading initially collapses and stiffens the polymer matrix. The decrease in molecular weight is slowed down due to the reduction of surface area from hydrolysis until the matrix architecture no longer accommodates the mechanical loading and begins to lose its integrity.

Bone is able to remodel *in vivo* under physiological loading,[31] and tissue-engineered articular cartilage needs a mechanical stimulation[32] to assimilate similar properties to natural cartilage. Based on this biological process, it is a requirement that the degradation and resorption kinetics have to be controlled in such a way that the bioresorbable scaffold retains its physical properties for at least 2 to 6 months (e.g., 1 to 3 months for cell culturing and 1 to 3 months *in situ*). Thereafter, the scaffold matrix can start losing its mechanical properties and should be metabolized by the body without a foreign body reaction after 12 to 18 months.[20] The mechanical properties of the bioresorbable 3-D scaffold–tissue construct at the time of implantation should match that of the host tissue as closely as possible.[33] It should possess sufficient strength and stiffness to function for a period until *in vivo* tissue ingrowth has replaced the slowly vanishing scaffold matrix.

In the case of constructs that are subject to extensive *in vitro* culture and implanted in a partially developed state, the intrinsic mechanical properties of the scaffold architecture guide the cell proliferation and differentiation only up to the phases where the premature bone or cartilage construct is placed in a bioreactor or is transplanted.[20] The degradation and resorption kinetics of the scaffold are designed to allow the seeded cells to proliferate and secrete their own extracellular matrix in the static and dynamic cell seeding phase (week 1 to 4), while the polymer scaffold gradually vanishes, leaving sufficient space for new cell and tissue growth. The physical support by the 3-D scaffold is maintained until the engineered bone or cartilage has sufficient mechanical integrity to support itself.

However, as the scaffold matrix degrades, contractile forces developed by the differentiating tissue can result in stresses that result in a significant decrease in the size of the tissue-engineered construct compared with the size of the original scaffold.[34] Different research groups have shown in a number of studies that a nonwoven mesh made of polyglycolide fibers and PLGA foams offers degradation and resorption kinetics suitable for constructs that are cultured *in vivo*. This work has

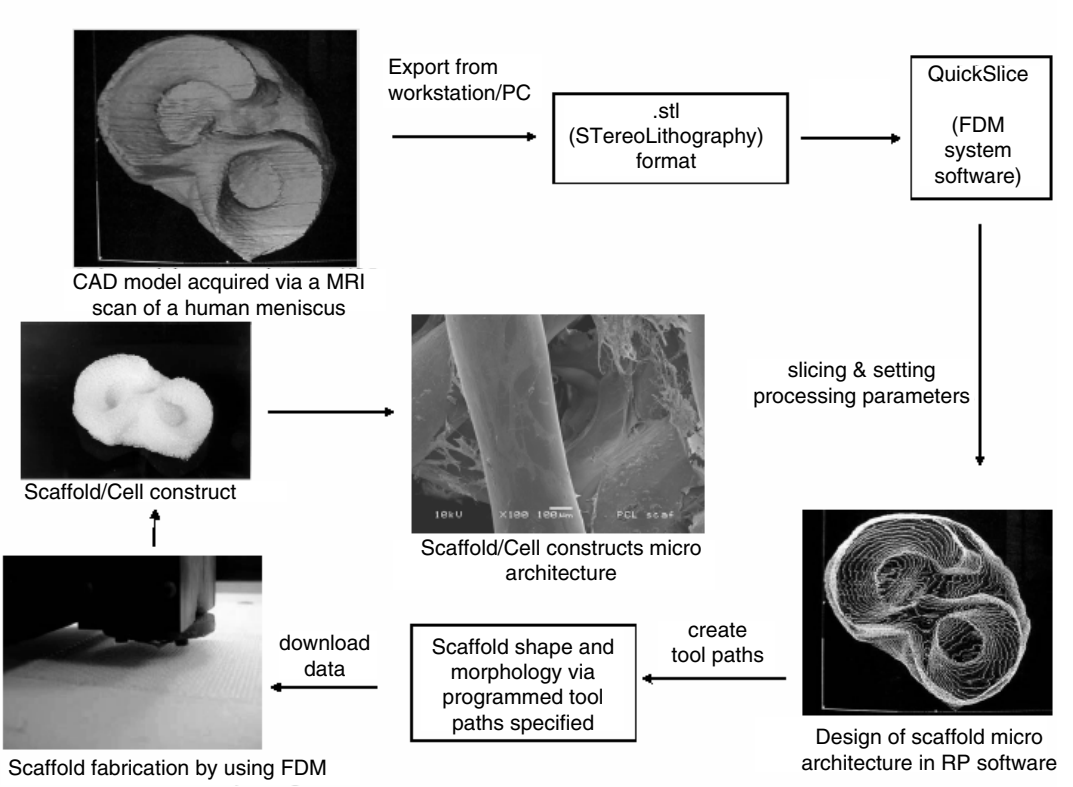


FIGURE 6.6 RP technologies allow the development of manufacturing processes to create porous scaffolds that mimic the microstructure of living tissue. The flow chart shows how a scaffold/cell construct of a human meniscus is created.

been reviewed by Freed.[19] The challenge for the *in vitro*–grown cell–tissue construct is to produce a final implant with similar mechanical properties to host tissue.

6.3 SCAFFOLD FABRICATION

Rapid prototyping and solid free-form manufacturing techniques have grown in popularity. These techniques offer ways of producing well-controlled, regular microstructures from a range of suitable scaffold materials and can be automated and integrated with imaging techniques to produce scaffolds that can be customized both in microstructure and overall size and shape for preparation of implants tailored to specific applications or even to individual patients (Figure 6.6). Some RP/SFF techniques are actually quite slow, whereas others can produce large quantities of formed scaffold quite quickly and lend themselves to large-scale production of scaffold materials. Most of these techniques offer good to average control over microstructures with pore wall resolutions on the order of 100 to 1000 micrometers.

6.3.1 Systems Based on Laser Technology

6.3.1.1 Stereolithography Apparatus (SLA)

The stereolithography apparatus (SLA), as it is commonly known, is often considered the pioneer of the RP industry. The first commercial system was introduced in 1988 by 3D Systems Inc., with the inventor C.W. Hull as one of its founders. Stereolithography is based on the use of a focused

beam of an electromagnetic radiation source — in 3D System's case, an ultraviolet (UV) laser, which is vector-scanned over the top of a bath of a photopolymerizable liquid polymer material. As polymerization is initiated by radiant energy, a first solid plastic layer will form at and just below the surface of the bath where the laser beam strikes. This first solid layer rests on a platform that is lowered into the resin (via an elevator system) such that the surface of the new first layer is just below the surface of the resin by a defined layer thickness. This laser-generated polymerization process is repeated for the generation of the subsequent layers by tracing the laser beam along the design boundaries and filling in the 2-D cross section of the model, layer by layer, until a plurality of superimposed layers forming the desired device is obtained. Once the model is complete, the platform rises out of the vat and the excess resin is drained. The model is then removed from the platform, washed of excess resin, and then placed in a UV oven for a final curing. The model can then be finished by smoothing the "stair steps."[35,36]

Typically, the photopolymer resins are mixtures of simple low-molecular-weight monomers capable of chain-reacting to form solid long-chain polymers when activated by radiant energy within a specific wavelength range. The commercial materials used by SLA equipment are epoxy-based or acrylate-based resins that offer strong, durable, and accurate models. This is also a major constraint for the use of the SLA technique for tissue engineering, as photopolymerizable biomaterials are hard to come by; these materials have to attain a required mechanical stability and prerequisite properties for biomedical applications. Even so, it can be expected that the range of materials could be extended to include metals, ceramics, and suitable composites. Due to the restraint of the liquid resin material, the SLA cannot fabricate overhanging structures without support. Usually, these supports have to be incorporated into the design of the main structure and integrated in such a way that after fabrication they can be removed without affecting the main model; one needs to keep in mind that the "green" assembly is usually brittle and weak before postprocessing.

As the laser point causes the resin to polymerize at the surface, this point-by-point material addition method enables a limited one-dimensional control that can produce detailed intricate structures such as controlled porosity. The standard SLA layer resolution is determined by the standard elevator layer resolution of up to 1.3 μm and laser spot size of 80 to 250 μm[35] (www.3dsystems.com). However, the resolution of the model would be further compromised after postprocessing due to curing and shrinkage. While there might be no problems with trapped materials, the trapped liquid resin would require hours to drain out before the postcuring is complete. Another significant resolution problem is due to the absorption and scattering of the laser beam, which only takes place near the surface. This produces a parabolically cylindrical voxel characterized by its horizontal line width and vertical cure depth; a single cured line would have a bullet-shaped profile. This is not necessarily a major limitation, but would result in a pronounced deformation when smaller and more intricate objects are made. Other disadvantages include the material's cost, its toxicity, and the fact that it must be shielded from light to avoid premature polymerization. The part is usually weak at time of removal and needs postprocessing for further curing.[35]

The most widespread use of SLA in the biomedical industry is presently limited to the creation of accurate models for surgical planning or teaching. However, there have been investigations into the fabrication of implantable devices and scaffolds using photopolymerizable biomaterials with the SLA method (internal communication). SLA provides a high degree of control for intricate devices as well as the incorporation of bioagents into them. Although limited to a photopolymerizable precursor, biocompatible acrylic, anhydride, and polyethylene oxide (PEO) precursor systems may be explored, as they are already in research or clinical stage, typically as curable bioadhesives or injectables. The variation of the laser intensity or traversal speed may be used to vary the crosslink or polymer density within a layer so that the properties of the material can be varied from position to position with the part.

In a recent publication, Cooke et al.[37] used a custom-mixed resin material consisting of poly(propylene fumarate) (PPF), diethyl fumarate (DEF) solvent, and bisacylphosphine oxide (BAPO) as

the photoinitiator, in the ratio of 140:100:1. The materials were mixed in a standard fume cabinet and attained a viscosity similar to that of the standard SLA resin. The SLA used was the SLA 250/40 from 3D Systems Inc. Cooke aimed to study the regeneration of critical size defects and designed a $\varnothing$ 50 × 4 mm plate. The part was fabricated and postcured for 2 hours in a UV oven. Cooke described the fabricated part as closely matching the geometry of the CAD design. Features such as holes, slots, and protrusions were satisfactorily maintained. As the part was built on support structures, some of the supports (< 10%) failed to attach to the build table and the part, resulting in dimples on the underside surface.

As a custom-made vat was used, Cooke attributed the manufacturing problems to the lack of adherence of the supports to the build platform and to the disturbance of attachments as the platform was relocated for the successive recoats and builds. The spacing and the diameter of the holes in the build table also affected the flow and spread of the resin. Another suggestion was to minimize resistance to the flow and spread of resin, as the PPF used was pure and of high molecular weight, with a high viscosity and surface tension. However, making the resin less viscous by adding more solvent would compromise the crosslinking process. Research is currently underway to reach an optimization of the resin viscosity and curing properties. Additionally, a higher degree of crosslinking would decrease the rate of degradation. In conclusion, Cooke's experiment successfully demonstrated the feasibility of using the stereolithography process to build and control 3-D multilayer parts made from a biodegradable, biocompatible resin. However, the real challenge for fabricating 3-D scaffolds for tissue engineering would be to attain an intricate architecture and high porosity as opposed to a plate.

6.3.1.2 Selective Laser Sintering

6.3.1.2.1 Laminated Object Manufacturing (LOM)

LOM is a process where individual layers are cut from a laminated sheet (e.g., paper) by a computer-controlled laser, after which the individual layers are bonded together to form a 3-D object. LOM has been used for fabrication of bioactive bone implants, using HA and calcium phosphate laminates. The undersurface of the foil has a binder that when pressed and heated by the roller causes it to glue to the previous foil. Once the parts have been built, the exterior of the slice is hatched to help the removal of the excess material, as opposed to fluid-based processes (e.g., the SLA process), where the interior is hatched. A disadvantage is the production of burnt edges due to the laser cut, which is not an issue with most applications, but creates unwanted and possibly harmful debris in biomedical applications. Material degradation in the heated zone may also occur. Newer technology has substituted the laser with a blade, and this might be applied in the biomedical field in the future.

6.3.2 Systems Based on Print Technology

6.3.2.1 Three-Dimensional Printing (3DP)

The 3DP technology was developed at the Massachusetts Institute of Technology (MIT).[38] This SFF technique utilizes a powder and a binder; the liquid binder is capable of binding and gluing the powder particles together. The 3-D printer constructs the 3-D model by first spreading a layer of fresh powder over a build platform. An "inkjet" print head prints or deposits the binder solution onto the powder bed. After the 2-D layer profile is printed, a fresh layer of powder is laid over it. The printing cycle continues and the layers merge when fresh binder is deposited, until the whole part is completed.[39] After the binder has dried in the powder bed, the finished component can be retrieved and unbound powder removed for postprocessing, if necessary. One such printer is the commercial 3-D printer from Zcorp, which utilizes either a starch-based powder or a plaster-based powder; both powders use a common water-soluble binder. After the component is completed, it can be infiltrated with wax or epoxy (www.zcrop.com).

3DP is capable of producing overhanging structures, which is not possible in some alternate techniques. While some SFF have limitations in manufacturing certain designs, such as the overhanging structures, the 3DP process is capable of overcoming that.[40] The solution lies in the layering of powders; as the layers are spread, there is always a supporting platform of powder for printing and binding to take place. Thus, as long as the parts are connected together, overhanging structures are of no difficulty. Likewise, internal pores (overhanging wall structures) can easily be created without worry of supporting materials below. However, one drawback of the powder-supported and powder-filled structure is that the open pores must be able to allow the internal unbound powders to be removed, if the part was designed to be porous. The resolution of the printer is basically limited by the specification of the nozzle size and the position of the control that defines the print head movement. Another factor is the particle size of the powder used, which simultaneously determines the layer thickness. Zcorp printers can achieve a layer thickness between 76 and 254 μm. The surface roughness and the aggregation of the powdered materials also affect the efficiency of removal of trapped materials.[40] Nevertheless, 3DP is highly accurate and is able to achieve any geometry, with complex micro- and macrostructure, composition, and surface texture control.[39,41,42] A chief advantage of the 3DP method is that it can be performed in normal ambient environmental conditions, such as temperature.

The versatility of using a powdered material is both an advantage and a constraint of the 3DP in that one must obtain micro-powder for the process. Most of the available biomaterials do not come in the required powder form,[43] milled PLA pellets under liquid nitrogen–chilled conditions to yield 75 to 150 μm, which was a tedious and time-consuming process as the efficiency in obtaining the micro-sized powders was low. Powders were stored in vacuum until usage. One group[44] also reported cryogenic milling to obtain usable PCL powder sizes; this same experience was shared by another group,[45] who used PLLA. Despite these restrictions, over the years the 3DP has become one of the most investigated and published SFF manufacturing techniques that researchers have developed for tissue engineering purposes and drug delivery devices. The following researchers have managed to employ the 3DP effectively for biomedical device applications incorporating biomaterials.

One group[44] reported studies in which the 3DP technique was adopted to investigate the manufacture of drug delivery devices. In this pioneering work conducted in collaboration, Wu and the inventors of the 3DP, Cima and Sachs,[38] used a nonautomated 3-D printer to fabricate and study the 3DP process in accomplishing interior local microstructure control and local composition of a component. The equipment was operated manually, from the raising and lowering of the build platform to the spreading of the powder. A single print nozzle (45 μm) was used in this working prototype. The materials used were poly(ε-caprolactone) (PCL) and polyethylene oxide (PEO), and they were arranged into two lots of 45 to 75 μm and 75 to 150 μm. The binder used was a chloroform–PCL solution. The drug release profile or degradation of the device was controlled by printing walls with different thickness and by the use of the two polymers, which have different degradation rates. Through this study, they have concluded that 3DP could offer several unique build strategies for obtaining zero-order release kinetics, an ideal situation for most drug delivery devices. A highly specific release profile was indeed achievable by the reproducible local microstructural control using this SFF.

One of the earlier works on fabricating tissue engineering scaffolds from 3DP[46] used PLGA (85:15) powder packed with salt (NaCl) and a suitable solvent to fabricate scaffolds. The scaffolds were ∅ 8 × 7 mm in shape with designed interconnected pores of 800 μm and micropores of 45 to 150 μm resulting from the salt leaching. Although not reported, the design porosity was estimated to be about 35%, but the overall scaffold porosity was reported to be 60%, a consequence of the salt particles.

Kim et al.[46] have concluded that the 3DP technique allowed the creation of polymer scaffolds with highly complex macro- and microarchitecture, and larger-sized highly porous devices could be produced compared with the previously limited discs. They have reported on the success of culturing hepatocytes using the scaffolds in both static and dynamic conditions. Thus, Kim et al. have effectively set a benchmark for tissue engineering scaffolds produced via 3DP for other researchers to follow.

In 2001, Zeltinger and coworkers[45] fabricated scaffolds via the TheriForm™ solid free-form fabrication process, which utilized a 3-D printer from Therics Inc. For the raw materials, they used poly(*L*-lactic acid) (PLLA) powder mixed with salt (NaCl) and chloroform as the binder. The PLLA was cryogenically milled with liquid nitrogen, after which both the polymer and salt powders were sieved into lots of less than 38, 38–63, 63–106, and 106–150 μm. Disc-shaped scaffolds (∅ 10 × 2 mm) were fabricated using two compositions of salt–polymer ratios, 75:25 and 90:10, and the four different lots of particle sizes. Printing was accomplished using a stencil process to control deposition of binder on the polymer powder. After printing the chloroform on the desired regions, the evaporation of the chloroform resulted in precipitation of the polymer around the salt particles, forming a continuous polymer–salt structure. Residual chloroform was extracted from scaffolds by placing them in liquid CO_2 at about 7˚C and 800 psi for 5 min. The vessel was vented for 120 min at a rate of 5–10 standard cubic feet per hour. The salt was leached from the scaffolds by immersing in 500 ml of deionized water at room temperature, rotating at 40 rpm for 3 h, and changing the water hourly.

Zeltinger and coworkers[45] claimed to have demonstrated that the TheriForm technology could form scaffolds with complex macro- and microarchitectural features. However, they used a stencil technique to control the printing process, which resulted in a stenciled macroarchitecture, and the microarchitecture was a consequence of the salt particles and independent of the TheriForm technology; both macro- and microstructures could easily be achieved by the salt-leaching technique incorporated with casting. In addition, by fabricating only a 2-mm-thick scaffold, they have demonstrated the critical limitation when resolving techniques related to salt leaching and solvent dependence. Consequently, they have also greatly undermined the potential and function of the SFF technology. As a result, the cell culture studies reported some problems with interconnectivity of the pores. The use of an organic solvent probably also posed a problem to the working environment and restricted the close monitoring of the fabrication process.

Lam et al.[42] have also made use of the commercial 3-D printer Z402 from Zcorp, one of the six licensees of the 3DP technology from MIT, for their investigations. A suitable biomaterial blend was formulated for the study, which was composed of 50% cornstarch, 30% dextran, and 20% gelatin. All are natural biomaterials: Starch is a polysaccharide produced by plants, dextran is a bacterial polysaccharide obtained from certain strains of bacteria and slimes, and gelatin is a polypeptide derived from naturally occurring collagen. This blend was designed to operate well within the parameters of the printer and was maintained at less than 100 μm. Distilled water was used as the binder. Five different cylindrical scaffold designs (∅ 12.5 × 12.5 mm) were produced using CAD software, with different pore interconnectivity. After fabrication on the 3DP, the scaffolds were postprocessed to enhance the strength and increase their resistance to water. The scaffolds were dried at 100°C, after which the unbound powders were removed. A series of infiltration and postprocessing methods were examined, and it was concluded that the method of infiltration with 6.9 ml of PLLA–PCL (75:25) copolymer solution — which involved drying, then immersing for 10 minutes in water, and finally drying again at 10°C — produced the strongest and most resistant scaffold.

The use of natural biomaterials along with water as the binder eliminated the problem encountered with organic solvents of creating a toxic manufacturing environment. However, this also created the predicament that the scaffold, because it was bound by water, was also water-soluble. This led to a lengthy postprocessing route to "waterproof" the product. Subsequently, Lam et al. have successfully characterized the scaffold physically, chemically, and mechanically,[42] although no cell-culture studies have been published. Lam et al.[42] have also pointed out another dimension to the products of the 3DP process: As they are all made from bonding powdered particles, the fusion of particles usually would not totally eliminate all the microspaces and gaps in between the particles. They have shown that scaffolds made from the 3DP process possess two types of porosities. The first is the designed interconnected porosity. The second is a porosity caused by the gaps between the fused particles, referred to as *microporosity.* This microporosity is likely to be created at random and will be critical in the degradation kinetics of the scaffold.

6.3.3 Advanced Manufacturing-Based Systems

6.3.3.1 Shape Deposition Manufacturing (SDM)

Marra et al.[47] reported the use of a shape deposition manufacturing (SDM) technique to construct osteogenic scaffolds based on blends of PCL and P(D)LGA incorporated with hydroxyapatite (HA) granules for bone tissue engineering applications. The group investigated the use of scaffold fabrication processes that allowed simultaneous addition of cells to the interior scaffold regions during the scaffold synthesis process. In this manufacturing process, the scaffolds were incrementally built up from thin, prefabricated cross-sectional layers of foams (approximately 1 mm thick). The foams were made using a solvent-casting and salt-leaching process as reported in the literature. They were then cut using a computer-numerically-controlled (CNC) process to generate each cross section, based on a 3-D computer-aided design (CAD) model.

Homopolymer discs as well as blends of 10:90 (10% PCL and 90% PLGA) and 40:60 (40% PCL and 60% PLGA) were prepared with incorporation of HA between 0 and 50% (w/w). The scaffold porosity was reported at 80% by controlling the amount of sodium chloride particles incorporated. The foam layers were stacked up to form the 3-D discs by mating the layers together with biodegradable or nonbiodegradable fasteners, including miniature barbs, sutures, screws, and nuts. The discs were mechanically assembled using sutures for *in vivo* experiments in rabbits. Each prefabricated layer was first seeded with cells and growth factors before final assembly. However, it was highly dependent on the quality of the prefabricated foams. The salt particle size used to create the micropores and the resultant scaffold pore size had not been reported. Furthermore, the assembly stage of the fabrication process has to be carried out such that there is sufficient pore-to-pore interconnectivity, otherwise the seeded cells would be trapped within each layer, inaccessible to external supply of nutrients.

6.3.3.2 Robotic Microassembly

The robotic microassembly technique is currently being investigated and developed at the National University of Singapore.[48] The principle of microassembling a functional tissue engineering scaffold is based on the same concept as assembling a structure using small building block units like Lego®. Building blocks of different designs would be first fabricated via lithography or other microfabrication technologies and assembled by a dedicated precision robot with microgripping capabilities with four degrees of freedom, resulting in a scaffold with the required material, chemical, and physical properties. A monolithic shape-memory-alloy microgripper was used to manipulate and assemble the unit microparts into a scaffold structure.

Presently, two elemental unit designs have been explored. The first is a 2-D planar micropart, $420 \times 420 \times 60$ μm^3 in size, and could be fabricated at a precision of 2 to 3 μm using plasma etching (oxygen plasma), UV LIGA, or injection molding. The second design is a 3-D cross-shaped building block with overall dimensions of $500 \times 500 \times 200$ μm^3 and a wall thickness of 60 μm; it is axis symmetric and stable. It may be produced using UV LIGA or injection molding. Both parts may be assembled by pushing a unit part onto another and stacking up a complete scaffold. By applying a suitable force, the microparts would stick together due to friction (Figure 6.7).

6.3.4 Extrusion Technology-Based Systems

6.3.4.1 Three-Dimensional Plotting

In this method, a dispenser head is controlled by a three-axis platform, typically a CNC machine or robot. The process generates an object by building microstrands or dots in a layered fashion. Depending on the type of dispenser head, a variety of materials can be used to build scaffolds. Silicone scaffolds have been tested with the system. Landers et al.[49] have presented a machine that

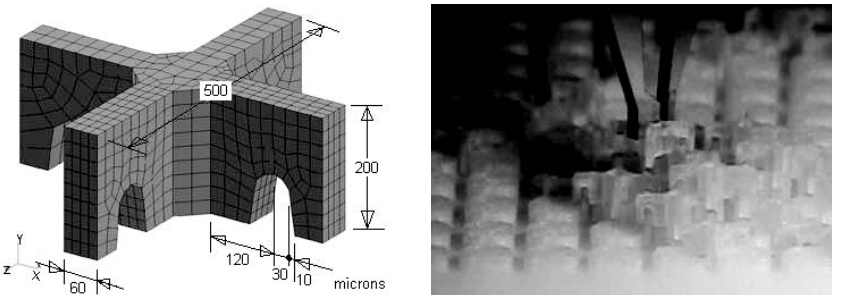


FIGURE 6.7 A group at the National University of Singapore describes the fabrication of microscopic Lego®-like building blocks to be used in the assembly of scaffold/cell constructs utilizing a high-precision robot. The distribution of growth factors and living cells within the scaffold can thereby be controlled in three dimensions of freedom so that a scaffold/cell construct with diverse biological properties can be realized. The fabrication of these microparts is challenging due to their small size ($0.5 \times 0.5 \times 0.2$ mm overall, 60 mm thickness) and complex three-dimensional shape. Another challenge is the requirement that the parts need to be stably fixed on the wafer but at the same time easily removed by the microgripper. (From Zhang, H. et al., *Proc. of IEEE Int. Conf. on Robotics and Automation*, Washington, DC, 2002. With permission.)

can make use of a wide variety of polymer hot melts as well as pastes, solutions, and dispersions of polymers and reactive oligomers. The advantage of such a system is its versatility. However, resolution is the primary limiting factor, determined by the size of the dispensing tip and material properties. Hutmacher's group developed a system similar to the one developed by the Freiburg group (Figure 6.8).

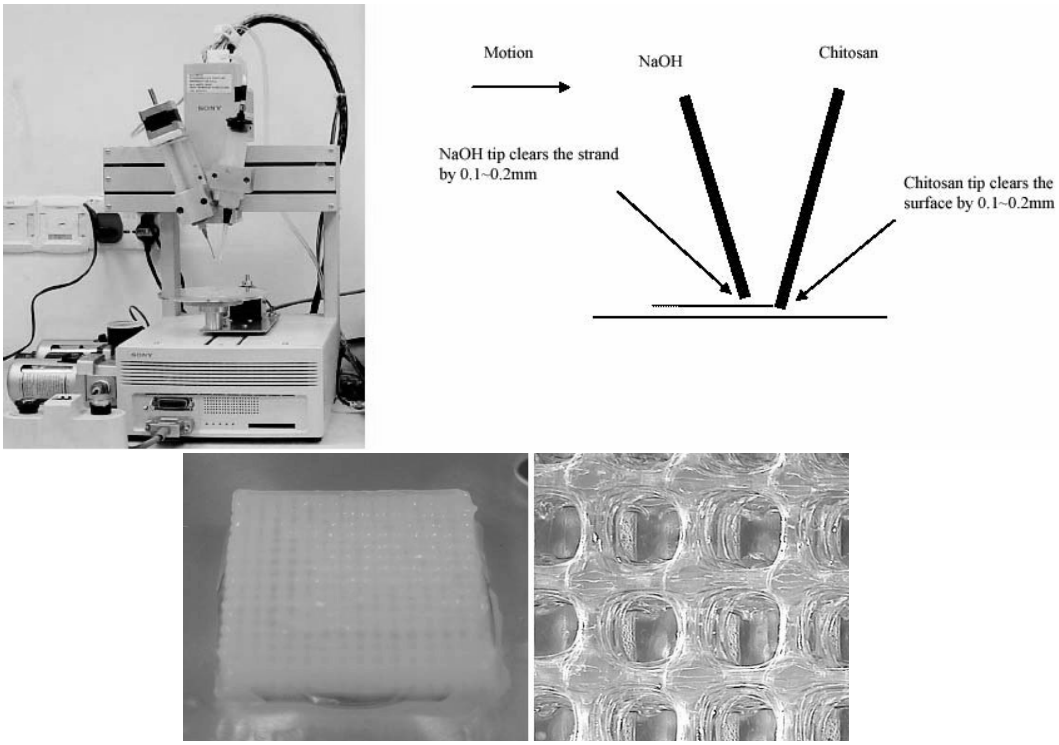


FIGURE 6.8 Chitosan scaffolds produced by a three-dimensional plotting process. A dispenser head is controlled by a three-axis platform, which generates an object, in this case a chitosan scaffold, by building microstrands or dots in a layered fashion.

6.3.4.2 Multiphase Jet Solidification (MJS)

The basic principle of the multiphase jet solidification (MJS) method involves the extrusion of a melted material through a jet-like nozzle. The MJS process is usually used to produce metallic or ceramic parts via a lost-wax method. Koch and coworkers[50] studied the use of the MJS process to build scaffolds made of poly(*D,L*-lactide). In this method, the modeling material, supplied as powder, pellets, or bars, was first heated to achieve a suitable viscosity, squeezed out through a nozzle by a pumping system, and deposited layer by layer. The molten material solidified when it touched a base platform or the previous layer. The extrusion head was mounted on an *x-y-z* table controlled by a computer system. After one cross section was produced in the *x-y* plane, the extrusion head moved in the *z* direction (between 0.05 and 0.5 mm) and the next layer was manufactured. The entire part was created layer by layer up to the top.

Three-dimensional hollow PDLA structures were fabricated this way based on design models programmed by in-house MJS software. Special structures were designed to tissue-engineer bone and cartilage. The structures had a reported pore size of 300 to 400 μm. Koch and coworkers thought that the ingrowth of human bone tissues into their implant structures would start at these levels.

However, there was no report on the detailed scaffold morphology using microscope analysis or on any mechanical study of the scaffold properties. There was also no follow-up report from the same research group in the use of the MJS process in tissue engineering since the first paper was published in 1998. Unlike the patented FDM method, to which the MJS process was highly similar, the latter was not commercialized. Because the MJS process is very similar to the patented fused-deposition modeling process, it has not been separately commercialized.

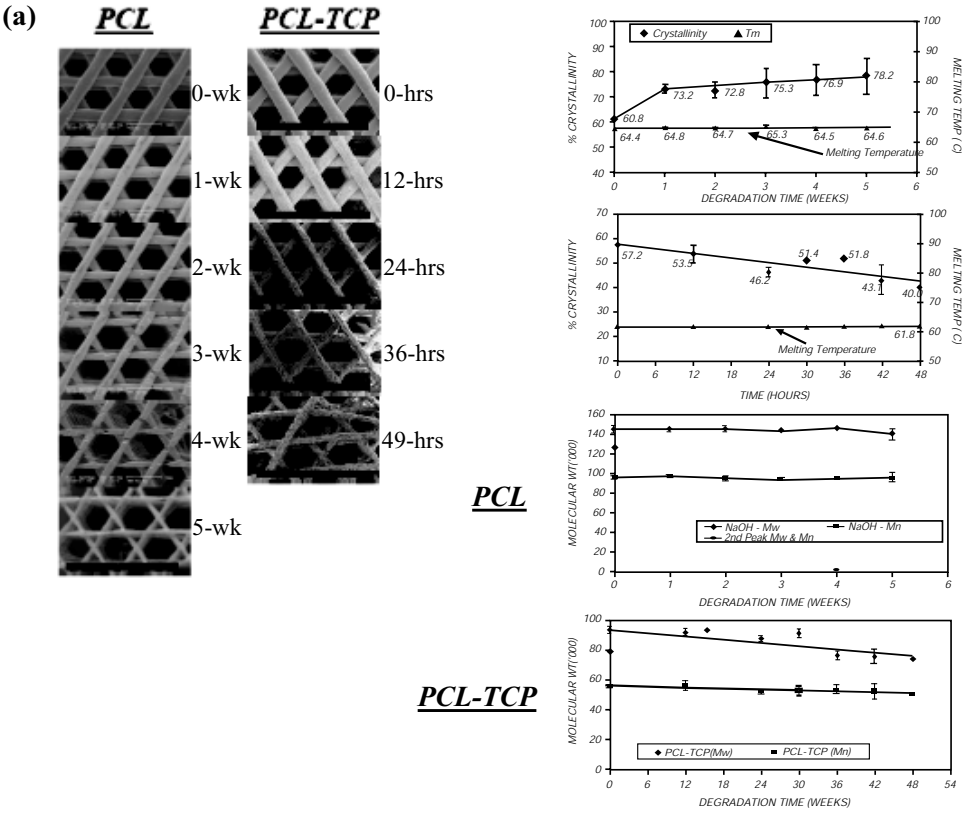
6.3.4.3 Fused Deposition Modeling (FDM)

A traditional FDM machine[51] consists essentially of a head-heated liquefier attached to a carriage moving in the horizontal *x-y* plane. The function of the liquefier assembly is to heat and pump the filament material (polymer, wax, or ceramic-loaded binder) through the nozzle onto the build platform. The spooled filaments are fed into the liquefier via a set of two feed wheels driven in counter-rotating direction by a small dc servomotor, which provides enough torque to the filament to act as a piston during the extrusion phase. The filament softens and melts inside the liquefier and then is extruded out of a nozzle located at the bottom end of the liquefier. The extruded material is laid down on a build platform following the contour tool path. In general, the contour (defining the inner and outer boundaries for each layer) is laid down first, after which the interior is filled. Once a layer is built, the platform moves down one step in the *z* direction to deposit the following layer. Parts are made layer by layer, and the layer thickness can vary from 25 μm to 1 mm.

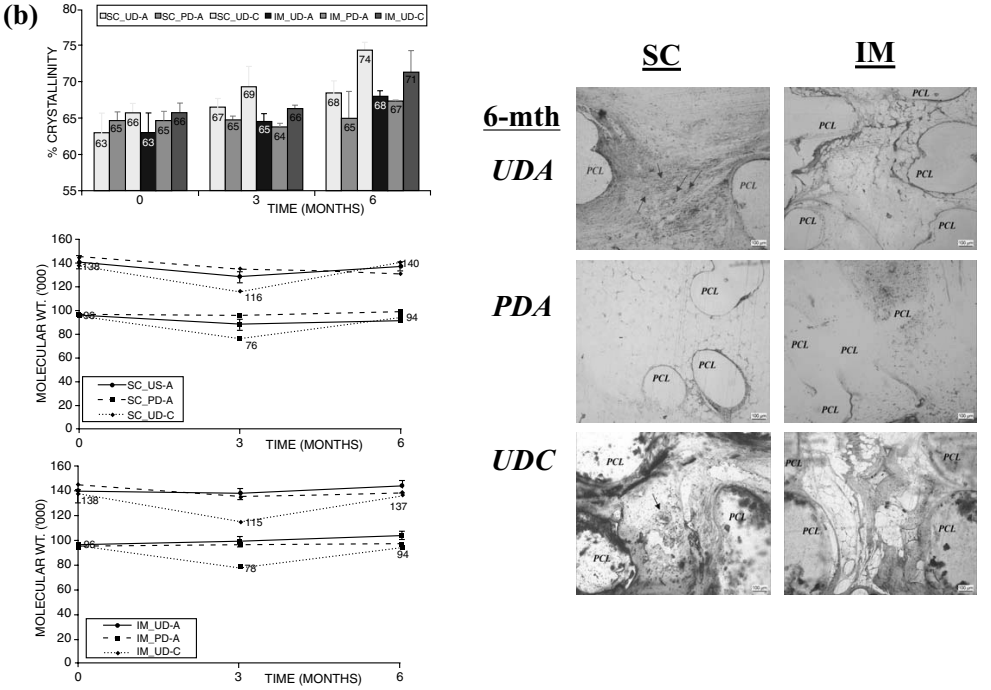
Currently, Hutmacher and coworkers (unpublished data) had been able to evaluate the parameters to process their so-called second generation of scaffolds for bone engineering based on PCL/TCP (Figure 6.9) and PCL/HA by FDM. They reported that FDM had allowed the design and fabrication of bioresorbable 3-D scaffolds with a fully interconnected pore network. Due to the computer-controlled processing, the scaffold fabrication was highly reproducible.

However, one of the disadvantages of the FDM process is the restriction on thermoplastic materials. The high temperature required to melt the material rules out the possibility of using any biological materials as base material or as additives. Furthermore, precise temperature control of the extrusion head is required to ensure consistent strand dimensions and adhesion.

FIGURE 6.9 (See figure on facing page.) (a) Recently, Hutmacher's group developed its second generation of scaffolds for hard tissue engineering. Degradation studies showed that a composite scaffold (PCL/TCP 80/20%) manufactured by FDM showed a four to seven times faster degradation profile when compared to PCL scaffolds (unpublished data). **(b)** Preliminary *in vivo* biocompatibility and degradation studies in a subcutaneous (SC) and intramuscular (IM) rabbit model showed that the composite scaffold induces only a minimal and clinically undetectable foreign-body reaction.



In vivo



6.3.5 Indirect SFF

The key to the versatility of the SFF manufacturing techniques is their ability to build the model from its basic raw materials. However, the major limitations also lie in its methodology of building and bonding raw materials together. For the case of the SLA technique, the raw material must be in a liquid form and must be photopolymerizable. The raw materials for the SLS and LOM must be able to melt and be severed cleanly, respectively; particles and layers must also be able to bond together based on the energy supplied. The 3DP powder and binder combination must be compatible and effectively adhere to the bulk material. Finally, the FDM can only use a thermoplastic material. These limitations on the building materials further restrict the list and availability of biomaterials that can be used to fabricate scaffolds using the SFF technology. Some researchers have begun to explore other options to exploit the macroscopic geometry and internal intrinsic architecture attainable by the SFF, along with its convenience and accuracy of duplication from medical imaging sources. One emerging method is to fabricate a negative mold base on the scaffold design and cast the scaffold using the desired materials, which may not be usable in an SFF setting. Thus, the "indirect" SFF techniques were born.[52]

Chu and coworkers[52] used the lost mold technique, combining the epoxy resin molds made by SLA (SL 5170 and SLA 250/40 from 3D Systems, respectively), based on 3-D scaffold designs generated from computer-aided design (CAD) software or other imaging techniques, and a thermal-curable HA-acrylate suspension as the raw or slurry material. After the molds were formed, the HA suspension was subsequently cast into the epoxy mold and cured at 85°C. The cured part was placed in a furnace at high temperature to simultaneously burn out the mold and the acrylate binder. Following the mold removal, the HA green body in the designed 3-D structure was sintered at 1350°C into a 3-D HA scaffold. Chu et al. created three HA scaffold designs with interconnecting pores, one for mechanical evaluation and two for *in vivo* tests. However, the fabricated scaffolds contained several fabrication inaccuracies. Pores that were originally square-shaped resulted in bullet-shaped patterns due to the SLA limitations. In addition, post-sintering dimensional changes resulted in changes in the vertical height of the channels of up to 40%, while other errors were minimal. The *in vivo* experiment demonstrated osteoconductivity and biocompatibility of the HA scaffolds in a minipig model, with up to 16% and 45% bone coverage after 5 and 9 weeks, respectively. They have also concluded that the SLA had a limited resolution of 150 × 320 μm curing effectiveness.

In their works published in 2003, Taboas et al.[53] and coworkers applied the indirect SFF method with conventional sponge scaffold fabrication procedures in creating a series of biomimetic scaffolds for multitissue and structural tissue interface engineering. They designed scaffolds of ∅ 8 × 8 mm with a porosity of 50%. The first step was to create the molds for the scaffold; this was achieved using wax and polysulphonamide (PSA), which were commercial materials for the Model Maker II 3-D printer from SolidScape Inc. The versatility of this machine was that both wax and PSA can be used as the modeling material or the support material, as there is a choice of removing either material by their respective means. With this setup, four molds were made. PSA molds were made by melting and subsequently dissolving the wax portion in Bioact®. Wax molds were obtained by dissolving PSA in acetone, while cement and ceramic molds were created by casting cement paste in wax mold and an HA–acrylic slurry in an inverse mold, respectively. The final ceramic (HA) mold was further burnt out and sintered.

With the molds made, four different casting routes were used to create scaffolds with different features. The first casting route used three sets of solvent casting with porogen leaching to create a scaffold with local porosity. They are the traditional salt leaching with salt (104 to 124 μm) and PLA (7.5%)–solvent combination and the emulsion-solvent diffusion method using a PLA–tetrahydrofuran and ethanol combination or snap-freezing technique. The second casting route called for simply solvent-casting PLA (25%) into the mold. The third casting route involved melt-casting PGA and PLA into a top–bottom composite scaffold. The fourth casting route produced a poly-

mer–ceramic (HA–PLA) composite by a process that involved melt casting and etching. All the final scaffolds were retrieved by the respective appropriate mold removal process involving melting, dissolving, or etching.

Taboas et al.[53] reported that the ceramic molds shrank by 50% in volume and that this could be compensated for via the design before the casting. The 3DP process generated grooved spacings. Generally, any imperfections or cracks in the molds were reflected in the final scaffold. The melt-cast scaffolds reveal small inclusions due to trapped air. They have successfully fabricated scaffolds with global interconnected pores ranging from 500 to 800 μm resulting from the prefabricated mold, and when local pores were created, they ranged from 10 to 300 μm subjected to the local pore crating method.

In conclusion, indirect SFF adds further versatility and detail in scaffold mold design. The previous restriction on casting was the inability of molds to produce complex geometry and internal architecture. Now, with indirect SFF, traditional casting processes with these SFF molds can meet the specific tissue engineering requirements, including mechanical integrity and customized shapes. Some highlighted advantages of indirect SFF include cost savings, as the material required for mold casting is substantially less and need not be processed into a dedicated form for any particular SFF process, such as processing into a powder for SLS and 3DP. In addition, indirect SFF allows the use of a much wider range of materials or a combination of materials (composites or copolymers) without the constraints of the SFF material requirement, such as breaking free of the limitations of a photopolymerizable polymer for SLA and a thermoplastic for the FDM. However, some drawbacks still revolve around this method including the resolution of the SFF method, as the cast model would inherit the errors, and defects from the mold, such as cracks and dimensional changes. In addition, a mold removal method must be developed to remove the mold while preserving the cast scaffold and desired properties.

A group at Carnegie Mellon University, along with their collaborators, is developing a novel technique to materialize the concept of creating vascularized living tissue grafts for direct implantation, using the principles of tissue engineering and SFF. They aim to infuse cells simultaneously as the scaffold is being synthesized in a layered manner. In order to achieve this, the scaffold fabrication processes must depart from the traditional involvement of heat and toxic chemicals.

The overview of their concept entails the fabrication of layered scaffold in a customized geometry derived by the use of clinical imaging data, which is processed and translated to the desired scaffold layer (~ 1 mm) by a computer-numerically-controlled (CNC) cutting machine. The material used for the scaffold has been proposed to be a specially formulated polymer–HA composite; the HA would possess varying surface and microstructural attributes embedded *in situ* within a biodegradable polymer backbone. The next step would involve seeding of cells onto each geometrical layer and assembling the cell–scaffold layers together using biodegradable screws, sutures, or fasteners. Finally, this combined cell–scaffold construct would be implanted.

The group has demonstrated the feasibility of the assembly concept, with a study conducted at a heterotopic site in a rabbit model. Five individual layers of porous polymer–ceramic composite measuring ∅ 12 × 1 mm were individually seeded with autogenous bone marrow cells, and the layers were assembled into a 3-D construct. These constructs, along with a control-layered construct, were implanted into the rectus abdominis muscle of the rabbit. After 8 weeks, histological analyses showed that the cell-seeded construct appeared as a whole graft while the control showed discernible discrete layers. The group reported more bone formation in the seeded implants than in the controls (www-2.cs.cmu.edu/People/tissue/front_page.html).

6.4 CONCLUSIONS

Although extensive literature on specific requirements for scaffold design and fabrication has been published, there is no ideal scaffold at present. A broad range of parameters within which successful

cell or tissue culture and remodeling can be achieved is dependent on the tissue to be engineered and the overall strategy of the interdisciplinary team. Given specific ranges of porosity and types of structure, some specific constraints exist with regard to mass transfer and requirements for vascularization, which are subject to ongoing research. In many ways, remodeling of a tissue-engineered scaffold–cell construct after implantation can be considered guided wound healing, and it can be concluded that for each notable tissue, most constructs become extensively remodeled as part of normal tissue repair process. Therefore, production of very precise structures or copies of biological tissue structures (e.g., trabecular structures, dermal collagen matrix, etc.) needs to be balanced with the biological constraints of wound healing.

RP/SFF techniques provide manufacturing processes that are capable of producing scaffolds from a range of suitable materials and to a range of architectures, morphologies, and structures within the design tolerances and parameters of the processing concept. Some of these techniques offer the right balance of capability and practicality to be suitable for fabrication of materials in sufficient quantity and quality to move holistic tissue engineering technology platforms into the clinical application.

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7 Biodegradable Composites for Biomedical Applications

N. M. Neves, J. F. Mano, and Rui L. Reis

CONTENTS

7.1 INTRODUCTION

There is a growing interest in the biomedical field in the use of biodegradable materials for temporary implants. The goal is to circumvent the need for a secondary surgery associated with the removal of nondegradable implants or the problems associated with the long-term body reaction to the materials used. Biodegradable materials, however, need to meet more stringent requirements than nondegradable materials. Key issues include the biocompatibility, the possibility of leaching toxic contaminants (residual monomers and stabilizers, among others), and the potential toxicity of degradation products and metabolic residues. Furthermore, the implants should ideally degrade at a rate that is compatible with the healing time of the tissue of interest.

Examples of such biodegradable products include resorbable sutures, some bone plates used for spine fusion, and intramedullary nails.[1–5] The resorbable sutures are required to hold stresses during wound healing and to progressively lose mechanical function that is to be transferred to the healed skin. In the later cases, the functional tissue being substituted by the implant is bone, the structural material that among other important biological functions provides support and articulation to the human body.

The variety of available and suitable biodegradable materials is still too limited to cover the wide range of materials properties needed for producing implants and other biomedical devices. Thus, considerable research effort is being put into the development of new formulations and into

the modification of existing formulations either by chemical modification or by composition. Some recent developments include the development of self-assembling technologies and materials that are able to, under certain conditions, define a hierarchical structure. Those materials can also be tailored so that the surface will provide an adequate environment for cell development as well as promote the synthesis of an extracellular matrix.[6]

Generally, the materials of biological origin are particularly well adapted to their functions. Three main features of the biological systems[7–10] have been proposed: the structure defined at the nanometer length scale, clear and well-defined hierarchical levels of organization, and the interactions at the surfaces. Self-assembly of nanoscale-size units can produce nested structures. The nested structures may also self-assemble, often with the help of the living cells. The nanometer scale structural arrangement leads to materials properties fundamentally different from those expected from the bulk properties of its constituents.[11] The second key feature is the hierarchical levels of organization. Many biocomposite systems have at least one distinct structural feature at the molecular, nanoscopic, microscopic, and macroscopic scales.[12] These levels are organized into a hierarchical composite system designed to meet a complex spectrum of functional requirements. Bone, crustacean cuticle, and wood are excellent examples. As synthetic composite systems increase in complexity, they are known to function at higher levels of performance. Intelligent materials and adaptive composite systems may result from this type of complex architectural arrangement. It has been shown[13] that certain hierarchical laminates maximize (or minimize) the effective elastic moduli (or dielectric constant or thermal expansion) of a composite material. However, the connection between hierarchical design and final properties still needs to be understood in real products. Furthermore, although the principle of hierarchical design has already been applied to some synthetic composites,[14] the smallest level of hierarchy has not yet been reduced to the nanoscale. The third key feature is related to the specific interactions developing at the interfaces. For example, the pearly nacre of mollusk shells consists of layered plates of calcium carbonate ($CaCO_3$) (0.5 μm thick) held together by a much thinner layer of organic material.[15] This structurally highly organized matrix both acts as an organic template inducing growth of specifically oriented ceramic crystals (epitaxially) and contributes significantly to the shell mechanical properties. A key component of this matrix is silk, a protein with remarkable mechanical properties. Whatever the nature of the bonding between levels, adequate adhesion is required for the system structural integrity. Similarly, in the assembly of synthetic products, various processing methods may be used simultaneously to assemble units from small to large, with desired interfaces and structures.

Synthetic (or man-modified natural) materials are mostly "passive" materials, able to keep function under varying external stimuli (stresses, humidity, or temperature), but unable to regenerate or to actively react to changing conditions of the environment. Conversely, bone is known to adapt its structure as a reaction to external varying stresses, resulting in the loss of bone mass in low-gravity conditions, as observed in space stations,[16,17] or the increase of bone mass associated with heavy sports activities.[18,19] Existing biomaterials are of course less adaptable than living tissues. Thus, their design criteria take as reference the least favorable set of acceptable conditions, causing the structures to be overdimensioned most of the time. The vision of materials that are able to regenerate, heal, gain, or lose mass following the environmental demands (including stress or other stimuli) would open new ways of building structures. An example is the self-healing composite materials being developed by White et al.[20,21] The challenge in that case was to design a composite containing a self-repair system not affecting the material's overall properties or performance. It would be able to sense damage and then react to that damage and initiate healing. Finally, it should restore the material's original properties (strength and stiffness, for example). This array of requirements was successfully achieved with a glass fiber-reinforced epoxy composite containing dispersed encapsulated healing agent (Figure 7.1). Upon crack damage of the composite, the crack will be guided to the weaker capsules containing the healing agent. The capsules break, allowing the healing agent to fill the crack space. The healing agent contacts the catalyst that is dispersed in the composite and promotes the polymerization of the healing agent.

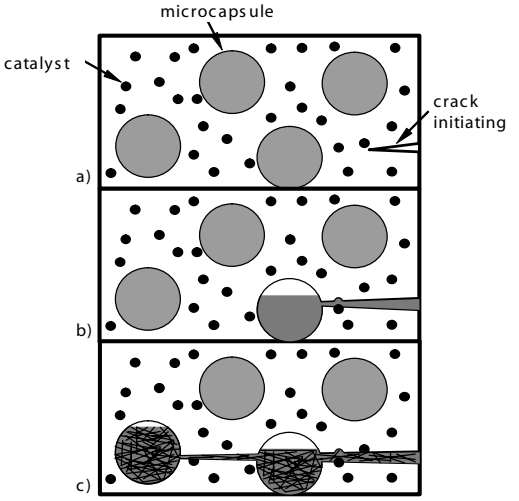

FIGURE 7.1 The self-healing concept. a) Crack initiates; b) crack opens the microcapsules, releasing the healing agent; c) the fluid contacts the catalyst, closing the crack. (Adapted from White, S.R. et al., *Nature*, 409, 794, 2001.)

When we compare biological materials with synthetic or (man-modified) materials, one finds that nature developed adaptive materials with hierarchical structure based in building blocks of the same type.

Nature did not choose passive materials with great diversity for each of the functional requirements existing in living tissues. Living tissues are based in molecules composed of a few basic elements of the periodic table and intertwined with living cell communities. This environment provides the necessary adaptive conditions for the materials.

The evolution in the biomaterials field has been made by increasing the variety of compositions and structures but mostly keeping the complexity at a low level compared with biological materials. Currently, it is still very difficult to build hierarchical structures that capture the essential attributes of biological tissues. Thus, the space of available solutions and properties of the existing suitable materials is discrete, and there are many regions of this space that are not covered by existing homogeneous materials.[22]

The lack of available biomaterials suitable for all the required needs has forced the development of composite biomaterials. Composite biomaterials are composed of two or more materials that are different in composition, structure, and properties, defining a continuous phase and at least one reinforcing phase. The reinforcement should be homogeneously dispersed in the continuous phase (commonly referred to as *matrix*) at the microscale, and at the macroscopic scale the material should behave as a homogeneous material. A distinctive characteristic feature of composites is that they can be designed, within defined limits, to tailor their mechanical or physical properties by judicious selection of their components.

The next sections of this chapter will cover the efforts being made to study and optimize biodegradable composites for biomedical applications. The materials with potential for use as matrix in biodegradable composites and the more promising reinforcements will be discussed. The chapter will conclude with an overview of the latest developments in the processing and applications of those systems.

7.2 COMPOSITES IN THE BIOMEDICAL FIELD

Polymer matrix composites are being increasingly studied[22–24] for different biomedical applications ranging from coatings to load-bearing implants. Examples of nondegradable polymers proposed as

matrices in biocomposites include polymethyl methacrylate (PMMA), polysulfone (PSU), polyetheretherketone (PEEK), and epoxy. The research concerning biodegradable composites has been mostly centered on the use of polylactic acid (PLA) and polyglycolic acid (PGA) and its copolymers, the first biodegradable polymers to obtain U.S. FDA (Food and Drug Administration) approval.[25,26]

Standard requirements for a polymeric material to be used in the biomedical field include fatigue resistance and resistance to aging in saline aqueous media, biocompatibility, dimensional stability, absence of migrating harmful additives, and the ability to be sterilized by standard methods without significant loss of properties. The biocompatibility requirement includes that the material and its additives should not elicit any toxic, inflammatory, or allergic reaction in the surrounding tissue.[27,28] However, when a biodegradable composite is used, new opportunities and challenges need to be faced. It is possible to control the degradation rate, eventually matching the pace of tissue healing. Both the matrix and the reinforcement should be biodegradable and resorbable. Furthermore, it is necessary to ensure that no harmful effect is produced by the degradation residues and metabolites.

Although natural fibers such as bamboo or silk[29] have been proposed for biomedical applications, currently the most studied reinforcement materials for bone-driven implants and tissue substitutes are bioactive fillers. Examples of those bioactive fillers are hydroxyapatite (HA),[30] tricalcium phosphate (TCP),[31] and bioactive glasses.[32] The composition of hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) is similar to the inorganic phase existing in mineralized bone and has high biocompatibility and bioactivity.[33] Bioactive glass is a special type of glass that has affinity with mineral bone, resulting in both mechanical reinforcement and bioactivity in polymer matrix composites.[34,35] Those reinforcements have been subjected to extensive research effort in recent years.

Composite materials' properties can be tuned by playing with the properties of both the matrix and the reinforcement (volume fraction of the discontinuous reinforcing phase, dimension and shape of the particles, particularly fibers, and its orientation).[36] In this way, it is at least theoretically possible to avoid the mismatch stiffness between the properties of commonly used metal implants and bone that causes the stress shielding effect and leads to bone atrophy.[37,38] One of the key parameters in controlling the successful design of polymer matrix composites is the control of the interface properties between the continuous phase (biodegradable polymer in this context) and the discontinuous phase. The interface can be improved either by chemical bonding[39] or by physical interlocking between the matrix and the reinforcement.[40] The goal is to obtain a good transfer of load from the continuous phase to the reinforcement.

Most of the composites are designed to target the desired mechanical properties, this being particularly relevant in the case of bone substitution. Thus, it is not surprising that the matrix materials that have been more extensively considered for that application are the ones with higher mechanical properties and the ones with renewed acceptance in terms of biocompatibility and biodegradability (e.g., the poly[lactic acid] polymers).

7.3 BIODEGRADABLE COMPOSITE MATERIALS

Many composite materials have been considered in recent years for use in bone substitution and regeneration strategies. Some reviews have been published that cover different materials in use and under development.[41–43] This section will review the most promising biodegradable materials both in terms of matrix materials and reinforcements considered for composites intended for biomedical applications.

7.3.1 Natural and Natural-Origin Biodegradable Composites

7.3.1.1 Collagen-Based Composites

Collagen exists as a family of isotypes that share a common triple-helical molecular structure providing these molecules with relatively high resistance against degradation.[44] The isotypes have different genetically determined amino acid sequences and can therefore be clearly separated from

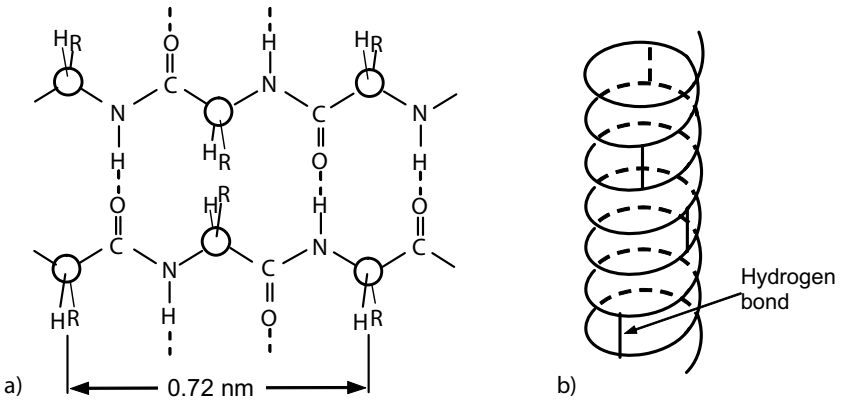

FIGURE 7.2 a) Flat sheet structure of a protein; b) helical configuration of a protein. (Adapted from Shu-Tung, L., *The Biomedical Engineering Handbook: Second Edition.* Bronzino, J.D., Ed., CRC Press LLC, Boca Raton, 2000.)

each other.[45] Up until now, 18 different collagen isoforms were identified. New collagen types (named from XIX to XXV) were recently reported in literature.[46–49]

Collagen molecules, like all proteins, are formed *in vivo* by enzymatically regulated stepwise polymerization reaction between amino and carboxyl groups of amino acids, where R is a side group of an amino acid residue.[50]

$$\left[-\overset{\overset{O}{\|}}{C}-\overset{\overset{H}{|}}{N}-\overset{\overset{H}{|}}{\underset{\underset{R}{|}}{C}}- \right]_n \tag{1}$$

The simplest amino acid is *glycine* (Gly) (obtained by substituting R by H in the chemical structure), where a hypothetical flat-sheet organization of polyglycine molecules can be formed and be stabilized by intermolecular hydrogen bonds (Figure 7.2a). However, when R is a large group as in most other amino acids, the stereochemical constraints frequently force the *polypeptide* chain to adopt a less constraining conformation by rotating the bulky R groups away from the crowded interactions. The most stable configuration is a helix, where the large R groups are directed toward the surface of the helix (Figure 7.2b). The hydrogen bonds are allowed to form within a helix between the hydrogen attached to nitrogen in one amino acid residue and the oxygen attached to a second amino acid residue. Thus, the final conformation of a protein, which is directly related to its function, is governed primarily by the amino acid sequence of the particular protein.

Collagen is a protein composed of three polypeptides (α chains), each having a general amino acid sequence of (-Gly-*X*-*Y*-)n, where *X* is any other amino acid and is frequently *proline* (Pro) and *Y* is any other amino acid and is frequently *hydroxyproline* (Hyp). The application of helical diffraction theory to high-angle collagen x-ray diffraction pattern[51] and the stereochemical constraints from the unusual amino acid composition[52] led to the initial triple-helical model and subsequent modified triple helix of the collagen molecule. Thus, collagen can be broadly defined as a protein which has a typical triple helix extending over the major part of the molecule. Within the triple helix, glycine must be present as every third amino acid, and proline and hydroxyproline are required to form and stabilize the triple helix.

Collagenous matrix represents about 90% of the bone organic material and approximately 50% in cartilage tissues, although the collagen types are different in those tissues. Collagens I, III, V, VI, and XII have been localized in bone in various subsets of the osseous histoarchitecture, while

adjacent joint cartilage contains collagens II, IX, and XI. With respect to bone tissue, collagen I is the major type of collagen, forming the main organic part of the osteoid. Collagen II is known to be the major collagenous component of hyaline cartilage. Collagen can be analyzed and identified either by isolation and chemical characterization using biochemical methods,[53] such as gel electrophoresis or liquid chromatography, or by immunological techniques using specific antibodies.[53] The first set of methods provides quantitative results, but no data are obtained for the topologic distribution of the collagens. The latter approach in combination with optical microscopy offers data about the specific site of a distinct protein. At present, immunohistochemistry represents the major tool for the detection and localization of different collagen types in thin sections of bone and cartilage tissue.

The rationale for the use of collagen matrix composites for the substitution and regeneration of bone follows from the fact that bone is a collagen/hydroxyapatite nanostructured composite.[12] The HA nanocrystals of bone are regularly aligned along the collagen fibers.[54,55] The nanostructure of bone forms because of the cooperative actions of both osteoclasts (osteoclasis) and osteoblasts (osteogenesis). The HA nanocrystals are dissolved by H^+ ions supplied via proton pumps of the osteoclast and the collagenous fibril is simultaneously decomposed by an enzyme acting in acidic conditions. However, the details of osteogenesis have not been clearly understood. In general, osteogenesis starts by the production and release of collagen fibrils from the cells. The fibrous collagen matrices combine to form bundles followed by calcification with the release of Ca^{2+} and HPO_4^{2-} ions or HA nanocrystals. Bone is thereby the extracellular matrix formed outside of cells; however, its structure could be constructed in the microscopic area locally regulated by the osteoblasts,[56] where the raw materials of bone can be spontaneously organized to a regular nanostructure. Thus, there have been attempts to produce materials having identical functionality and nanostructure.

Itoh et al. proposed in a series of works[57–60] the use of collagen as a base for the preparation of composites reinforced with hydroxyapatite with an emphasis on promoting a self-organization mechanism by focusing on the regulation of pH and temperature in an aqueous solution, trying to mimic bone properties. The biological reactions of the composite in bone defect were examined by means of animal tests.[59] The HA/collagen composites obtained had a similar nanostructure to bone in which the *c*-axes of blade-shaped HA nanocrystals 50 to 100 nm in size were aligned along collagen fibers up to 20 μm in length. The mechanical properties of the composite were 40 MPa in bending strength and 2.5 GPa in elastic modulus. The observation of histological sections showed that the composite was incorporated into the remodeling process of bone and was resorbed by osteoclastic cells, and new bone was formed by osteoblasts after the resorption. The application of those composites for an artificial vertebral body was studied in dogs. The study concluded that the presence of recombinant human bone morphogenetic protein can improve the rate of fixation into the native tissue. The implants collapsed after 13 weeks of implantation,[60] the results being comparable with previous studies using HA ceramic implants.

Mehlisch et al.[61] implanted a mixture of HA particles and collagen and reported its high biocompatibility. Miyamoto et al.[62] prepared collagen-reinforced self-setting HA cement and indicated its higher biocompatibility and improvement of mechanical properties.

TenHuisen et al.[63] prepared a HA/collagen nanocomposite using crystallization of HA on collagen fiber from the acid–base reaction with $CaHPO_4$ as an HA precursor.

Many other researchers[64–80] have prepared and reported HA–collagen composites with similar techniques.

The mechanical properties of various collagen fibers and tissues with high collagen content are shown in Table 7.1. The table illustrates the fact that collagen itself has modest mechanical properties, particularly in wet state. The reinforcement with hydroxyapatite leads to a significant improvement of the mechanical properties (2.5 GPa). Data in Table 7.1 indicates that the HA reinforcement obtained in the bone tissue is much more effective than in currently developed collagen/HA composites.

TABLE 7.1
Mechanical Properties of Collagen Fibers and Tissues with High Collagen Content[57,81–84]

	Stiffness (GPa)	Strength (MPa)	Ref.
Crosslinked extruded rat tail tendon collagen isolated fibers	0.3–0.5	25–50	81
Rat tail tendon	1.2	115	81
Rat tail tendon fibers, wet	0.5–0.6	33–39	82
Rat tail tendon fibers, dry	2.1–2.7	360	82
Human patellar tendon	0.2	—	83
Cortical bone	17	130 (tension)	84
HA/collagen composite	2.5	40	57

Data from Kikuchi, M. et al., *Biomaterials*, 22, 1705, 2001; Gentleman, E. et al., *Biomaterials*, 24, 3805, 2003; Kato, Y.P. et al., *Biomaterials*, 10, 38, 1989; Atkinson, T.S., Ewers, B.J., and Haut, R.C., *J. Biomech.*, 32, 907, 1999; Lakes, R., in *The Biomedical Engineering Handbook*: *Second Edition*, Bronzino, J.D., Ed., CRC Press LLC, Boca Raton, 2000.

Although exhibiting very interesting mechanical properties, the most severe shortcoming of collagen as a matrix to design composite implants is its animal origin (mainly bovine and porcine, whose immunogenic response and disease transmission to human tissues is a problem). Human-derived collagen is also viable, its use being hindered by economical reasons. The major antigenic reaction sites in collagen are the telopeptides located at both ends of the collagen molecule. Treating the collagen with pepsin, it is possible to remove those telopeptides (atelocollagen) and reduce significantly the antigenicity.[85]

7.3.1.2 Hyaluronic Acid-Based Polymers

Glycosaminoglycans (GAGs) consist of linear arrangements of repeating disaccharide units, usually including a uronic acid component (such as glucuranic acid) and a hexosamine component (such as *n*-acetil-*d*-glucosamine).[86]

The largest GAG, hyaluronic acid (hyaluronan), is an anionic polysaccharide with repeating disaccharide units of *n*-acetylglucosamine and glucuronic acid, with unbranching units ranging from 500 to several thousands. Hyaluronic acid can be isolated from natural sources or via microbial fermentation.[87] Because of its water-binding capacity, dilute solutions of hyaluronic acid are viscous.[86]

Like collagen, hyaluronic acid can be chemically modified to reduce its water solubility and to increase its viscosity. Crosslinking can produce higher-molecular-weight complexes (in the range of 8 to 24×10^6) or a molecular network (gel).[88,89]

The relative ease of isolation and modification and its superior ability to be shaped into solid structures has made it the preferred GAG in medical device development. In fact, hyaluronic acid is not antigenic, eliciting no inflammatory or foreign body response, making it desirable as a biomaterial.[86] Gels and films made from hyaluronic acid have been used for prevention of postsurgical adhesion.[90–92]

Hybrid composites of collagen–hyaluronic acid–hydroxyapatite were prepared by drying dispersion precipitates of the components.[93] The aim was to develop a hybrid type of composite able to adhere to both hard and soft tissues, with good cohesion strength, and without disintegrating when immersed in body liquids. Both very good dispersion of the HA particles and a better swelling behavior was observed in the hybrid composite than in collagen–HA composites prepared with the same method. The bending strength of the composites was also evaluated, confirming the better interface properties of the components of the hybrid composites (5.4 kPa) than the Col/HA coun-

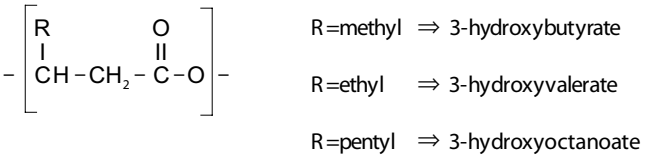

FIGURE 7.3 Chemical structure of poly-3-hydroxybutyrate.

terpart (3.6 kPa). Biocompatibility was assessed by cytotoxicity tests, and no adverse reaction was observed in the cells.

7.3.1.3 Polyhydroxyalkanoates

Polyhydroxyalkanoate (PHA) polyester biopolymers are a broad family of microbial storage polymers, which accumulate as granular inclusions in a wide range of bacteria.[94] Over the past 30 years, the PHA family has emerged from the first identified PHA, poly-3-hydroxybutyrate (PHB) (see chemical structure in Figure 7.3), to encompass a family of materials comprising over 130 hydroxyacid monomer types.[95,96] The ability to modify the material properties of PHAs by controlling their monomeric composition, molecular weight, and final physical form as either a plastic resin or an amorphous latex suspension has attracted significant industrial interest.

Initial efforts focused on a single polymer type, poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV), which was sold under the trade name Biopol from ICI. This material was targeted primarily as a biodegradable alternative to polypropylene, although it was more expensive. Since that time, however, the production and evaluation of several members of the broader family have uncovered a wide range of material properties with potential applications across the industrial spectrum.[97,98] For example, by controlling the ratio of the two monomers in the copolymer poly-3-hydroxybutyrate-co-4-hydroxybutyrate, a range of material properties can be achieved that range from rigid thermoplastic to elastomeric.[99]

The past two decades have seen major advances in the understanding of the PHA metabolism, and this has been reviewed extensively elsewhere.[97,100,101]

The production of PHA by plants is a subject of great research and industrial interest; the interested reader can find a review of the most recent advances in this subject elsewhere.[94]

The P(HB-HV) polyesters are semicrystalline polymers with very high degrees of crystallinity (60 to 80%).[102–104] The degree of crystallinity is one of the most important basic parameters characterizing semicrystalline polymers. Crystallinity affects virtually all other bulk properties of the polymer including mechanical, physical, thermodynamic, and optical properties. From a pharmaceutical perspective, crystallinity will influence polymer properties such as rate and mechanism of degradation, drug compatibility and drug diffusion, and therefore drug release.

When extracted from different bacterial sources, the homopolymer PHB has essentially similar properties. Furthermore, the properties of PHB extracted from *Ralstonia eutropha* are thought not to vary with the nutrients used for fermentation.[102] Molecular weights of the polymer obtained have been reported to change with the bacterial source, but there have been suggestions that the variation in molecular weight may be due to the extraction process used. The different extraction processes are thought to lower the polymer molecular weight to varying extent. A report by Scandola et al.[105] showed that PHB extracted from *Rhizobium* sp. using 0.1 *M* HCl had a molecular mass of 60.000, but when extracted with acetone, PHB samples had molecular masses of the order 1000 kDa.

PHB is a crystalline thermoplastic with a melting temperature range of 160 to 180°C, depending on the molecular weight and thermal history of the sample.[102] It can be melt- or solution-processed into films, sheets, and fibers.[102]

Hydrolytic degradation of PHB *in vitro* proceeds to the monomer hydroxybutyric acid.[106] This acid is a normal constituent of blood and, like acetoacetate and acetone, is one of the three ketone

bodies that are produced endogenously by the process known as ketogenesis. It is therefore thought that PHB will be well tolerated *in vivo*. However, limited information is available on the hydrolytic degradation of P(HB-HV) copolymers or other PHAs.

The *in vitro* degradation of PHB is slower than that of polylactide systems.[107,108] Pouton et al.[108] reported that studies of the mass loss from thin solvent-cast films (85 μm) at 37°C and pH 7.4 suggested that the half-life of these PHB films was about 152 weeks. Rate of mass loss from films of P(HB-HV) copolymers studied appeared to be more rapid, but there was no obvious rank correlation between degradation rate and HV content over the range 0 to 30 mol% HV. The kinetics of mass loss over a 50-week period approximated to zero-order, at least until there was substantial disintegration of the film, after which it was not possible to retrieve sample for weighing. Water uptake into the polymers was too low to be measured precisely (less than 0.01%), which led the authors to conclude that *in vitro* degradation of P(HB-HV) polymers proceeded by a surface erosion mechanism.[88] This was confirmed by molecular weight analysis of degraded polymer samples by nonaqueous size exclusion HPLC. The molecular weight of PHB remained unchanged throughout the 50-week degradation period in which the polymer films incurred a 15% mass loss.

The potential of those materials in bone tissue replacement has been proposed in different works.[102,109,110] The homopolymer offers potential advantages over the currently used materials in bone replacement therapy in that its mechanical properties are in the range of interest for matrices to be used in biodegradable composites. Furthermore, both the polymer[111] and a composite of PHB–7% PHV and a soluble phosphate-based glass reinforcement[112] were shown to have piezoelectric properties similar to bone. Those properties enhance the applicability of those materials for bone and cartilage regeneration.

Galego et al.[113] studied compression-molded composites of P(βHB-co-βHV) samples containing different amounts of hydroxyvalerate (HV) — 0, 8, 12, and 24% — and hydroxyapatite. Composites were prepared from as-received powders PHAs/HA using 30 wt% of HA after homogenization at different composition and compression-molding the mixture at approximately 5°C above the melting point of PHA. The results show modulus of up to 2.75 GPa and tensile strength up to 67 MPa at an elongation at break of 2.65%.

The low level of *in vivo* biodegradation of those materials needs to be improved, eventually by chemical modification or by compounding, to achieve degradation rates compatible with bone healing and regenerative medicine.

7.3.1.4 Starch-Based Biodegradable Blends

Thermoplastic starch is obtained by destructuring starch and blending it with other polymers. Starch has been blended with a range of synthetic polymers such as ethylene–acrylic acid, polyvinyl alcohol, ethylene–vinyl and ethylene–vinyl alcohol copolymers, ethylene-co-vinyl acetates, cellulose acetate and other cellulose derivatives, polycaprolactone, polyhydroxybutyrate, and other polyhydroxyalkanoates.[114] The synthetic phase can constitute between 15 and 70% of the weight of starch in the blend.

It has been shown that starch-based polymers can be degraded by specific enzymes such as alpha-amylase, beta-amylase, alpha-glucosidase, and other debranching enzymes.[115–118] The accessibility of enzymes to the starch sites depends on the synthetic component of the blend, on the efficiency of the mixing process of the starch with the synthetic phase, and on the degradation media used.

Conventional injection-molded composites of starch-based blends and HA were studied by Reis et al.[119,120] The largest mechanical properties of composites based in starch compounds for biomedical applications were reported by Sousa et al.[121] This work proposed the use of shear-controlled orientation in injection molding (SCORIM®) to induce anisotropy of mechanical properties of composites of starch/ethylene–vinyl alcohol blends reinforced with hydroxyapatite. The results showed that the SCORIM process leads to self-reinforcement of the matrix and that the HA

particulate reinforcement tends to get aligned along the composite axis. Tensile modulus of 7 GPa and tensile strength of 40 MPa (50 wt% of HA) were reported in the same study.

Starch-based blends were also reinforced with bioactive glasses. A blend of starch with ethylene–vinyl alcohol copolymer was reinforced with Bioglass®.[122] The materials were compounded and further injection molded using conventional technology. The 40-wt% Bioglass composites showed tensile modulus of 3.8 GPa and tensile strength of 38 MPa and excellent degradation behavior with retention of mechanical properties after 90 days of immersion in simulated body fluid. The bioactivity was also assessed, and a Ca-P layer was formed after only 6 days of immersion in simulated body fluid.

7.3.2 Synthetic Biodegradable Composites

7.3.2.1 Polycaprolactone (PCL)

Based on a large number of tests, PCL is currently regarded as nontoxic and biocompatible material. The Capronor system, a 1-year implantable contraceptive device, has undergone Phase I and Phase II clinical trials in the U.S. It is interesting to note that in spite of its versatility, PCL has so far been predominantly considered for controlled-release applications. However, in the last few years, PCL has also been widely proposed[123–124] for use in tissue engineering scaffolding.

Only a few research groups proposed composites based in PCL. The main reason for this is that the mechanical properties of PCL are not among the larger mechanical properties for a biodegradable polymer (0.6 GPa of tensile modulus).

Walsh et al.[125] proposed the use of HA–PCL composites prepared by a vacuum infiltration of monomer into porous HA blocks, followed by polymerization. A significant improvement of the compressive strength was obtained by this procedure (from 9 MPa to 37 MPa). The hydrophobicity of the PCL composite was shown to be suitable for cell attachment.

Corden et al.[126,127] proposed a method to produce long fiber-reinforced PCL composites using *in situ* polymerization, a variant of the resin transfer molding (RTM) technology used with thermosets. The preliminary studies report that the use of vicryl fibers (a low-modulus fiber) did not improve the properties of the matrix. They reported results showing the inverse variation of the tensile modulus of PCL with the molecular weight (in the range 30.000–70.000). The results in terms of wetting of the fiber meshes indicate that the fibers are well dispersed and immersed in the PCL matrix, making this process promising for other biodegradable composite systems able to be processed by *in situ* polymerization.

7.3.2.2 Poly(α-hydroxy esters)

Poly(α-hydroxy esters), such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), or their copolymers, poly(*D,L*-lactic-co-glycolic acid) (PLGA), are among the few synthetic polymers approved for human clinical uses, including those for small load-bearing applications.[128,129] The different ways of producing PLLAs, such as polycondensation, ring-opening polymerization, chain extension, and grafting, have been extensively reported.[130,131] Such works also give details on a variety of general properties of such polymers. The main features of PGA, and especially its application for devices in trauma and bone surgery, were reviewed by Ashammakhi and Rokkanen.[132] The most important characteristic of such poly(α-hydroxy esters) is their biodegradability, where their ultimate products (lactic acid and glycine for PLA and PGA, respectively) are nontoxic and are transformed into water and carbon dioxide in well-defined metabolic pathways. The underlying degradation mechanisms comprise a random, bulk hydrolysis of the ester bonds in the polymer chain, catalyzed by the ends of the carboxylic chains that are produced during the ester hydrolysis.[133] PLA is much more hydrophobic than PGA due to the additional methyl group in the structure of PLA. Therefore PGA degrades much more quickly (a few weeks[134,135]) than PLA, which can remain stable for 1 year[136] or more, depending on its degree of crystallinity. Copolymers of PLA and PGA

do not have interpolated properties of the pure components. For example, copolymers containing equal ratios of PGA and PLA degrade faster than pure PGA. The degradation in semicrystalline polyesters proceeds preferentially within the amorphous regions because of a higher rate of water uptake than the crystalline regions. The degraded segments could then diffuse and give rise to recrystallization; this increase of crystallinity during hydrolytic degradation can be detected from the whitening of the specimens.[137]

By itself, PLLA has interesting mechanical properties with an approximate tensile modulus of 3 to 4 GPa, flexural modulus of 4 to 5 GPa, tensile strength of 50 to 70 MPa, flexural strength of 100 MPa, and a strain at break of about 4%.[130,138,139] The mechanical properties of PLLA, however, may vary with molecular weight and crystallinity.[140] For PGA, the tensile modulus and strength can reach 6 to 7 GPa and 60 to 100 MPa, respectively, with a strain at break between 1.5 and 20%.[136,137,141] Again, such values are highly dependent on the molecular weight and crystallinity. However, for many orthopedic applications, such as the fixation of fractures and osteotomies or the use of interference screws for ligament repairs, the mechanical properties of such materials are insufficient. Composite systems based on poly(α-hydroxy esters) are being developed to enhance their mechanical performance or other properties, such as biocompatibility or bioactivity.

7.3.2.3 Ceramic Reinforcement

Most of the ceramics used as poly(α-hydroxy esters) filler are bioactive, such as calcium phosphate particles (hydroxyapatite [HA] or tricalcium phosphate [TCP]), as they help in promoting bone bonding properties.[142] Ceramic particles will also increase both the modulus and the strength of the resulting composites, being suitable for orthopedic applications, both in compact and porous forms. Moreover, the biocompatibility could be enhanced with ceramic particles that induce an increased initial flash spread of serum proteins compared to the more hydrophobic polymer surfaces.[143] Furthermore, foreign body reaction due to the release of acidic degradation products could also be minimized by the buffering effect of the basic resorption products of HA or TCP[144,145]; the ceramic can act as hydrolysis barrier, delaying the degradation of the polymer.[146] The auto-generated increase of local acidity due to degradation, for example, of PLA could enhance solubility of the ceramic, which could be used in new bone formation.[147] In this context, it should be mentioned that the chemical nature of the inorganic filler influences the general properties of poly(α-hydroxy esters). For example, studies on the acidic/basic features of calcium compound blended in poly(*D,L*-lactic acid-co-glycolic acid) (PLGA) concluded that the hydrolytic degradation of PLGA decreased with increasing basicity of the filler[115]; in that study, 30% of calcium dihydrogen phosphate, calcium phosphate, or calcium carbonate was blended with the polymer and studied after different immersion times (up to 8 weeks) in phosphate-buffered saline solutions.[148]

HA is the most-used ceramic in such composites, which are mainly prepared by incorporating the ceramic into a polymeric solution. The resulting gel suspensions of HA may then be dried under vacuum. The resulting solid composite may be shaped using different processing techniques. Composites can also be obtained by mixing HA particles with *L*-lactide before the polymerization.[146] An appealing list of references assigned to the different ways of preparing such composites may be found elsewhere.[149]

Detailed biodegradation and biocompatibility of the HA–PLLA composite was assessed by Furukawa and coworkers,[150] who studied the mechanical and histological properties of rod implants in rabbits (both subcutaneous and intramedullary). It was concluded that the degradation is faster in the case of uncalcinated HA (u-HA) than with calcinated particles (c-HA). The noninflammatory response of the tissues confirmed the bioactive behavior of the implants.[150] New bone formation was observed 2 weeks after implantation, especially in the formulation with highest HA content.[151] Direct bone contact with the composites, without fibrous encapsulation, was also detected by SEM. Another work[152] confirmed the bone-bonding ability of such composites, where the loads required to detach plates fixed on the surface of the bilateral tibia cortices in rabbits were measured up to

25 weeks after implantation. At any implantation time, the bonding strengths in the composites were always greater than for the pure PLLA implants. Finally, such composites containing 30 or 40% (w/w) HA were implanted in the discal femur of 21 rabbits, and the morphology of the specimens was fully characterized 2 to 4 years later.[153] The results clearly demonstrate the need of such long-term experiments in the evaluation *in vivo* of new biodegradable materials. After 3 years, trabecular bone bonding was effective in the rod; by the third year, the implant shrunk and its surface started to collapse, and finally, by the fourth year, total bone encapsulation was observed, together with further shrinkage. During this time, the molecular weight decreased more than two orders of magnitude. The c-HA–PLLA implants showed good osteoinductivity, but with less shrinkage and infiltration of osteocytes. Parallel studies confirmed that PLLA–HA composites exhibit positive tissue response and good osteointegration[154] after implantation of screws during 4 and 8 weeks in the distal femur of sheep. *In vitro* studies on PLLA–HA systems were also performed by Ma et al.,[155] where composites and pure PLLA scaffolds were seeded with osteoblasts and cultured up to 6 weeks. New tissue was formed more homogeneously in the porous composite than in the PLLA scaffold, and with larger osteoblast survival. The authors concluded that the incorporation of HA enhanced the osteoconductivity in PLLA scaffolds.[155]

The incorporation of HA into PLLA improves considerably both the stiffness and strength of the polymer. The modeling of the mechanical properties in HA particulate–reinforced PLLA matrix composites was investigated with finite-element analysis, where both the particle content and shape were analyzed.[156] Experimental data obtained by dynamic mechanical analysis on PLLA–HA composites (obtained from PLLA of two different molecular weights and different HA contents), processed by compression molding, showed a linear increase of stiffness with increasing percentage of the filler.[157] However, the improvement of the mechanical properties depends strongly on the processing method employed. One of the PLA–HA composites showing highest mechanical properties was developed by Shukinami and Okuno.[158] The initial bending strength of 280 MPa exceeds the bending strength of cortical bone (120 to 210 MPa); this strength could be maintained above 200 MPa up to 25 weeks after immersion in phosphate-buffered saline solution. Moreover, the modulus could reach 12 GPa,[158] one of the highest levels of stiffness reported in bioactive polymers. Such composites were obtained from precipitation of a PLLA–dichloromethane solution, where small granules of uniformly distributed unsintered HA microparticles (average size of 3 μm) can be obtained.[158]

Sintered HA exhibits low absorption kinetics. Examples of ceramics with completely absorbable and bioactive characteristics are nonsintered HA, tetracalcium, octacalcium phosphate, and especially TCP.[159,161] A particular ceramic used to reinforce polylactides was found to have three- or fourfold higher *in vitro* solubility than α-TCP,[162,163] in which the obtained composites presented suitable degradation characteristics and interesting mechanical properties, in the range of cancellous bone. Absorbable ceramics may also be suitable in the production of cell–scaffold constructs for tissue engineering applications. An interesting example is the production of osteochondral scaffold that has different properties along its structure.[164] The cartilage region exhibits higher porosity, and the cloverleaf-shaped bone portion consisted of an L-PLGA–TCP composite, designed to maximize bone ingrowth while maintaining adequate mechanical properties.

Other calcium-based inorganic filler may be used as reinforcement of bioabsorbable polymers, such as calcium carbonate. It seems that the introduction of such particles (5 and 10 wt%) in PLLA may increase the hydrolysis degradation, at least in the presence of proteinase K.[165] Such filler may induce some bioactive character in the composites: The introduction of 30 wt% in PLA improved the modulus by 3.5 to 6 GPa (ca. twice the modulus of PLA), and a bonelike apatite layer was formed after immersion for 1 to 3 days in simulated body fluid (SBF) solution.[166]

Another example of bioactive inorganic filler is Bioglass®. Jerome and coworkers developed some poly(*D,L*-lactide) foams coated and impregnated with Bioglass. Processing was done either with a slurry-dipping technique with a pretreatment of the porous structure in ethanol[167,168] or by using a thermally induced phase separation process.[169,170] Tests in SBF revealed a clear bioactive behavior of the composites,[167–170] and seeded human osteoblasts cells attached and spread on all

surfaces,[168,170] indicating that such scaffolds may be adequate for bone tissue engineering. Lu et al.[171] showed that a 45S5 bioactive glass also induces bioactivity in polylactide-co-glycolide porous composites. Moreover, the composite possessed better mechanical properties than homogeneous polymer. Moreover, the addition of such bioactive glass granules supported osteoblasts-like morphology, stained positively for alkaline phosphatase, and supported higher levels of type I collagen synthesis than tissue culture polystyrene controls. Therefore, the composite has better osteointegrative potential when compared with polylactide-co-glycolide.

There is a growing interest in using layered silicates, such as montmorillonite, to reinforce polylactides. The challenge in those composites is to achieve intercalation of the polymer chains within the aluminosilicate layers (with ca. nm thickness). The use of surfactants has been proposed to lower the tendency to obtain agglomerates of the nanoparticles. The large surface area of contact between the matrix and the filler is believed to enhance the load transfer to the ceramic. Organic modification of montmorillonite is very common to improve the final properties of the nanocomposites. For example, *bis*-(2-hydroxyethyl)methyl (hydrogenated tallowaskyl) ammonium cations affects greatly the thermal stability of the composite.[172] Similar studies were performed in porous PLLA systems, where it was found that a small addition of montmorillonite (5.79 vol%) increased by 40% the tensile modulus.[173] The introduction of the nanoplates also decreased both the glass transition temperature and the degree of crystallinity in PLLA, leading to an increase of biodegradation rate. The solution intercalation method was used to produce nano-scale composite of PLA and both montmorillonite and fluorinated mica by Chang et al.[174] The latter composites exhibited better tensile properties. Better interaction between the polymer and the clay may be obtained if the polymer is grafted and grows directly onto the platelets' surface, as it was done with the PLA–montmorillonite system.

Polylactides may be reinforced with natural fibers. Flax fibers (30 and 40 wt%) were added into PLA and processed by extrusion and compression molding.[175] Although improved mechanical properties could be obtained, the interfacial adhesion still needs to be optimized. Jute fiber mats were also used to reinforce PLA by a film stacking technique.[176] Again, improved mechanical properties were observed, but voids between the fiber bundles and the matrix were detected. Those results showed that there is some potential for the use of natural fibers for PLA reinforcement and that there is still a need to improve the interface between the polymer and the fibers. Kenaf fibers seem to have a strong interface with PLLA.[177] Composites with 70 vol% of this fiber were found to have better mechanical properties than those of the kenaf sheet and the PLLA film themselves. The use of natural fibers as reinforcement of polylactides has been mainly proposed generally for applications requiring biodegradable polymer composites. Specific further studies need to be done to evaluate the potential of those composites for biomedical applications.

PLLA and PGA fibers exhibiting structures with a high level of orientation can be produced by mechanical deformation using polymer processing methods, such as oven drawing, zone drawing, zone annealing, die drawing, hydrostatic extrusion, or rolling. For example, by melt-spinning, fibers of PLLA can present 390 to 1800 MPa of tensile strength and 6.5 to 9.3 GPa of tensile modulus.[178–182] By solution-spinning, PLLA fibers can reach 560 to 2300 MPa of tensile strength and 9.6 to 16 GPa of tensile modulus.[178,183] PGA can also be spun into the fiber form, when the molecular weight is 20,000 to 145,000.[184] The sintering of such fibers at high temperature and pressures produces composite devices (rods, screws, tacks, plugs, arrows, or wires) in which the polymer matrix is reinforced with the self-reinforced material.[185–187] Such self-reinforced (SR) materials exhibit a significant improvement of the mechanical properties relative to the corresponding isotropic materials. Pohjonen et al.[188] reported that injection-molded PGA, sintered SR-PGA, and hot-drawn PGA rods with 2 mm diameter presented bending modulus of 7, 10, and 13 GPa; bending strengths of 218, 260, and 330 MPa; and shear strengths of 95, 192, and 260 MPa, respectively. Very good initial properties could also be observed in SR-PLLA screws, with bending modulus of 7 GPa, bending strength of 200 MPa, and shear strength of 110 MPa.[187] The shear strength decreased to 65 MPa (76 MPa *in vitro*) and 35 MPa (80 MPa *in vitro*) after 12 and 24 weeks of degradation *in*

vivo conditions, respectively. This demonstrates the more aggressive environment experienced by implants *in vivo*.

The degradation time of the implants may have implications in the tissue reactions when in contact with the products of the degradation. If the degradation is fast, the degradation products may not have enough time to be absorbed, due to poor vascularization or low metabolic activity. For example, PGA implants have been found to produce fluid-filled sterile sinuses with subsequent drainage, due to an increase of osmotic pressure or pH.[189,190] This could happen 8 to 16 weeks after surgery.[191] As PLLA implants degrade much slower (SR-PLLA may take up to 6 years to resorb completely), they are more tolerated by the organism, i.e., they induce less inflammatory response. A comprehensive revision of the inflammatory reaction in animals and humans to different materials was compiled by An et al.[192]

SR composites can be used in a variety of applications, such as in bioabsorbable fracture fixation or in other orthopedic surgery applications. They have been used since 1985, and the number of annual surgeries with such materials has exceeded 300,000.[191] Such systems can be used in glenoidal rim fractures; fractures of the proximal and medial condyle of the humerus; fractures of the lateral humeral, femoral, and tibial condyle; fractures of the olecranon, radial head, and distal radius; fractures of the hand, metatarsal bones, and phalanges of the toes; fractures of the femoral head and neck; fractures of the patella; and displaced ankle fractures.[192–194] Other uses of bioabsorbable fixation of bone include osteotomies, arthrodeses, and other reconstructive surgeries as reviewed by Rokkanen,[193] which included applications in orthopedic surgery and traumatology in children. Bioresorbable knitted stents were also developed from single SR fibers made out of poly(α-hydroxy esters).[161] PLGA stents lost ca. 35% of their initial weight at 11 weeks, together with a complete loss of their compression resistance strength.

These bioabsorbable implants can be combined with drugs or other bioactive substances that facilitate or accelerate tissue healing and they have themselves osteostimulatory effect. Tielinen et al.[195] examined the effect of adding a transforming growth factor — β1 (TGF-β1) polypeptide — into SR-PLDLA pins, which where implanted in the rat distal femur next to a bone defect filled with a viscose cellulose sponge. After 3 weeks, more fibroblast-rich mesenchymal tissue was observed inside the sponge in rats treated with TGF-β1, exhibiting larger amounts of new periosteal bone in the bone defect as well.

Other substances may be added to SR composites, such as radiopaque filler (barium sulfate powder), using different extrusion and drawing processes.[196] It was shown that the loss of intrinsic viscosity occurred at the same rate for filled or unfilled fibers. Bioactive ceramics (e.g., TCP or HA) can be added into SR composites to improve the mechanical properties of the pure polymer.[197,198] Typically, the flexural modulus increased from ~ 6.5 GPa for the case of pure polymer to 7 to 8 GPa for the case of the composites. On the other hand, the flexural yield stress increased from ~ 65 MPa for the unfilled material to 70 to 80 MPa for the 70% by weight HA content composite, and 80 to 100 MPa for the 70% by weight TCP composite.

7.4 BIOACTIVE REINFORCEMENTS

The bioactive materials are able to induce or modulate a specific positive biological activity.[199] For bone implants, the biological activity of interest is bone bonding. This may be defined as the establishment, by physical or chemical processes, of continuity between the implant and the bone matrix. Such bone formation may start both at the materials surface and at the surrounding bone tissue. Bioactive ceramics either are very similar to bone apatite or have the ability to form a calcium phosphate (CaP) layer on its surface when implanted that will promote bone bonding.[199] Besides the hydroxyapatite (the major inorganic constituent of human bone), other types of calcium phosphates, such as fluorapatite or tricalcium phosphate (TCP) and biphasic HA–TCP ceramics, are some examples of materials that also show a bone-bonding behavior and can be used as filler in biodegradable polymeric matrices.

Bioactive glasses, of which Bioglass is the most widely used, also enable bonding to bone. When implanted, these glasses can promote the formation on its surface of a bonelike apatite layer that will avoid the traditional fibrous encapsulation, allowing the creation of a continuous bone–glass interface.[200] The most typical bioactive glasses are based on Na_2O–CaO–P_2O_5–SiO_2 systems.[201] Other alternatives incorporating B_2O_3, CaF_2, MgO, Al_2O_3, among others, have also been proposed.[202] All these alternative bioactive glasses can exhibit a bone-bonding behavior. However, the kinetics of the calcium phosphate formation is rather dependent on the material's composition. The formation of a titanium or silica gel at the material's surface can also produce a bioactive behavior in certain materials.[199] In particular, the role of hydrated SiO_2 has been shown to be essential.

7.5 MELT-BASED PROCESSING OF COMPOSITES

Melt processing of biodegradable composites presents a number of challenges worth addressing in this section. The biodegradable character of the materials requires some degree of water absorption. Most of the biodegradable matrix materials being reviewed here are very sensitive to hydrolysis and are prone to thermal degradation. Polymer thermal degradation leads in most cases to hydrolysis and oxidation of the materials, causing breakdown of polymer chains. In some cases, the thermal degradation can promote reticulation and increase of viscosity of melts (e.g., polyethylene).

The production of composites requires a very good dispersion of the reinforcement agent, ensuring that the particles are perfectly surrounded by the matrix and not as agglomerates.[203] The wetting of the reinforcing particles, when not successful, can create local stress concentrations and lead to premature failure of the composite. The previous specifications are conflicting since efficient processing requires good mixing and homogenization of the melt without large temperature gradients. Frequently, melt processing of biodegradable materials is highly sensitive and unstable in the processing.

Traditional discontinuous thermoplastic-based composite materials are processed in two steps. The first step consists of compounding and pelletizing in an extruder. The second stage is the shaping stage, which will be responsible for the geometry to be produced. The geometry is the key parameter that determines the processing technology to be used in the shaping stage. Continuous section geometries will be produced by extrusion, three-dimensional nonhollowed geometries are injection molded, and hollow geometries are either blow molded or rotationally molded (rarely used to produce biomedical composites).

Polymer-processing technologies are based in the control of the viscosity of the melts. The processing temperature is set in the equipment and controlled by electrical heater bands. The mixing and homogenization is produced by the shearing stresses induced by the rotation of a reciprocating screw (both in extrusion and in injection molding). Shear stresses also generate heat by internal friction and can result in local thermal gradients within the materials during the plasticizing inside the barrel. The residence time of the material inside the barrel is a key parameter in avoiding thermal degradation. It is necessary to prevent any agglomeration of material inside the barrel to maintain low residence time.

Furthermore, the flow of polymer melts always results in some degree of anisotropy. The mechanism for development of orientation is as follows. The shear stresses cause preferential directions of alignment of the polymer melt during flow, particularly inside the mold or in the extrusion head. The subsequent fast cooling in constrained geometries (required to control the dimensions of products) can freeze the orientation of polymer chains, particularly in the outer skin of products, causing either molecular orientation if the matrix is amorphous[204] or aligned crystalline structures in semicrystalline polymers.

In the case of composites, if the particles are perfectly spherical (such as glass microspheres), the only possible source of anisotropy is an uneven dispersion of the particles. However, when the particles are approximately spherical, some degree of anisotropy can appear. This seems to be the case when hydroxyapatite and calcium phosphate particles are used as reinforcements.[121] Addition-

ally, if the particles of reinforcement are brittle, they may undergo some size degradation process during production, this being an eventual source of variation in the mechanical properties.[203]

It is important to highlight that biodegradable polymers have typically high susceptibility to thermal degradation during processing and frequently very narrow processing windows. Furthermore, any moisture content during processing can lead to hydrolysis and loss of molecular weight (e.g., PLA).

7.6 FINAL REMARKS

The search for biodegradable composites that are able to substitute for biological materials is an open field of research. Despite the great research effort already put forth, the vision of biodegradable materials able to restore the function and promote the regeneration of biological tissues is still ahead of us. Furthermore, the promise of materials that not only substitute for biological tissues, but also are able to actively adapt to varying conditions allows us to expect a great future from this research field in parallel with other areas such as regenerative medicine in general and tissue engineering in particular.

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8 Development of Bioactive Composites Based on Biodegradable Systems for Bone Replacement Applications

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CONTENTS

8.1 INTRODUCTION

In order to develop materials for bone replacement, it is fundamental to understand the structure and properties of the original hard tissue to be replaced. The development of new bone substitute materials has been a major challenge in recent years for many research groups,[1–7] since the bone is a highly complex hierarchical structure at both macro and micro scales.[8,9] At the micro scale, the bone apatite reinforced collagen forms individual lamella that range in size from the nm to μm scale, while at the macro scale, interstitial bone is composed by osteons ranging in size from the μm to the mm.[8,10] This hierarchically organized structure has an irregular, yet optimized, arrangement and orientation of the components, making the bone material heterogeneous and anisotropic.[1] More details on this can be found in Chapter 24 (A. J. Salgado et al.) in this book.

Bone consists of an organic framework (mostly collagen) in which mineral (carbonated apatite) plate-shaped crystals are dispersed.[11,12] Type I collagen constitutes approximately 95% of the organic matrix; the remaining 5% is composed of proteoglycans and numerous noncollagenous proteins.[12] The mineral phase is nanocrystalline apatite, structurally disordered, and compositionally nonstoichiometric with variable amounts of carbonated (CO_3^{2-}), fluoride (F^-), citrate ($C_6H_5O_7^{4-}$), and hydroxyl (OH^-) substitutions.[1,11,13] Bone is a complex living tissue in which the extracellular matrix is mineralized, which confers marked rigidity and strength to the skeleton while still maintaining some degree of elasticity.[5] Therefore, the bone tissue can be viewed as a two-component composite material composed primarily of collagen and mineral, hydroxyapatite, in the form of needlelike crystallites precipitated along the collagen fibrils.[14–16]

By means of combining an osteoconductive ceramic with a biocompatible polymer, it could be possible, in principle, to improve the biocompatibility and osteointegration of bone substitute materials and to develop implants with improved mechanical and bone-bonding properties.[4,5]

Moreover, this type of combination is expected to be a successful strategy for overcoming the difference in mechanical properties between bioceramics and natural tissues, turning such materials into valid alternatives to overly stiff metallic implant materials. In fact, one of the major problems in orthopedic surgery is the mismatch of stiffness between the bone and metallic implants due to the large difference in stiffness between bone (E = 7–30 GPa)[1,17] and metals (E = 100–200 GPa).[1,18] Hence, in the load sharing between the bone and implant, the amount of stress carried by each of them is directly related to their stiffness. Thus, bone is insufficiently loaded compared to the implant, and this phenomenon is called "stress-shielding" or stress protection.[19–21] It has been recognized that matching the stiffness of implant with that of the host tissues limits the stress-shielding effect and produces desired tissue remodeling.[19–21]

As a consequence, an ideal load-bearing orthopedic implant material must match as closely as possible bone mechanical behavior, especially its stiffness.[22] Over the past decade, there has been considerable interest in the development of polymer-based composites to be used as biomaterials for bone and joint replacement. There are several reasons for the development of polymer composite biomaterials, as for instance, the absence of fatigue failure, the corrosion and release of metal ions such as nickel or chromium, which may cause loosening of the implant; patient discomfort; and severe allergic reactions typical of metallic implants. Another reason for the development of polymer composite biomaterials is the typical low fracture toughness of ceramic materials, which makes them an unacceptable choice for load-bearing applications on their own.[19,21,23] The use of composite materials is a way of combining the strengths of the parent phases, minimizing undesirable characteristics.

Bonfield et al.[24] introduced the original concept of a bioceramic reinforced polymeric composite, using cortical bone as a template, by proposing a bioactive composite based on a high-density polyethylene (HDPE) and hydroxyapatite (HA) filler. This composite, designated as HAPEX™,[24–26] was designed to have specific bone analog mechanical properties and bone-bonding properties due to the osteoconductive character of HA.[21,26] Since its introduction, HAPEX has been successfully applied in the medical field.[27,28]

Moreover, the idea of combining a bioactive ceramic with a fracture tough phase, such as a polymer, to produce a composite with mechanical properties analogous to those of cortical bone and a bioactive character has been investigated in the last few years.[19,25,28] Since then, many composites have been proposed for biomedical applications,[2,3,19,22,29] including the development of polyethylene–Bioglass® composites.[30,31]

Bioglass–HDPE composites are being developed as a soft tissue-bonding material, and they exhibit a high degree of bioactivity and rapidly bond with the surrounding tissue. However, Bioglass–HDPE composites exhibit lower mechanical properties than equivalent HDPE–HA materials.[30]

The reason for choosing Bioglass as a filler arises from its osteoinductive potential.[20] This bioactive glass has the capacity to form a mechanically strong bond with bone through a biologically

active hydroxycarbonate apatite (HCA) layer formed at its surface that is chemically and structurally similar to the mineral phase of bone when they are implanted.[32–34] Furthermore, the analysis of the bone–implant interface revealed that the presence of hydroxyapatite is one of the key features in the bonding zone.[34] So, in other words, the essential requirement for an artificial material to bond to living bone is the formation of a biologically active bonelike apatite layer at its surface.[35–37]

However, the low strength of a monophase bioactive glass such as Bioglass, even with a high index of bioactivity, is an obstacle for its use in load-bearing clinical applications.[38]

Although Bioglass has excellent biochemical compatibility (bioactivity), which is a very important quality for artificial bone, this glass has, like all bioactive ceramics, a flexural strength and fracture toughness that is less than the typical bone values and a stiffness that is much greater than that of the bone.[38] This means that most bioactive materials have a less-than-optimal biomechanical compatibility when used in load-bearing applications.[39] An approach to solve this problem is the development of mechanically strong bioactive composites.

The ability of Bioglass of certain compositions to bond to soft tissues, as well as to bone, makes it a prime candidate for the reinforcing of a polymeric matrix, providing toughness and ductility to the resultant composite.[30,31] The strength of the bond is generally equivalent to, or greater than, the strength of the host bone, depending on test conditions.[20]

As a way to produce bioactive composite with controlled degradation behavior, several composite systems have been studied in the past few years; most of these systems combine biodegradable polymers matrices with bioactive ceramics.[4,40–43]

Biodegradable polymeric or composite surgical materials retain their tissue-supporting properties for given lengths of time (typically days, weeks, or months) and are gradually degraded biologically into tissue-compatible components, which are absorbed by living tissues and replaced by healing tissues.[44] However, one aspect that is very important for biodegradable materials is their biocompatibility. This means that the material must degrade without eliciting an unresolved inflammatory response or extreme immunogenicity or cytotoxicity.[45–47]

Devices obtained with biodegradable matrix composites present great advantages[21,23,44,48–51] and are the best alternative for the temporary internal fixation of many different kinds of tissue damage for the following reasons. One is the fact that during the early stages of tissue healing (as of bone, tendon, muscle, skin, etc.), the biodegradable implant holds the healing tissue in place. With the passage of time, the implant decomposes gradually and the stresses are transferred gradually to the healing tissue, avoiding the traditional stress-shielding effects associated with the use of very stiff materials. The other reason is the fact that biodegradable surgical devices do not require a removal operation, which is a substantial benefit both economically and to the patient being treated.

Materials that degrade slowly and predictably in the human body are useful in several medical applications, especially those serving temporary function such as sutures, bone fixation devices, applications related to reconstructive surgery, scaffolding for cells that recreate damaged or diseased organs, and controlled-release drug delivery devices.[23,44,49,52,53] Materials used in such applications include synthetic and natural polymers, ceramics, and ceramic-based composites.[54]

However, it has not yet been possible to develop new materials combining high bioactivity with a mechanical performance analogous to those of natural bone and a suitable degradation behavior.

In the last 8 years, starch-based polymers have been proposed[53,55,56–61] as alternative biomaterials for orthopedic applications. These materials combine a degradable behavior with an interesting combination of mechanical properties.[56–59] They are also able to exhibit a bioactive character through the incorporation of bonelike inorganic fillers, such as hydroxyapatite (HA) or bioactive glasses and glass–ceramic[5,62–66] powders. Additionally, it has been shown[62,67–71] that these materials can comply with the biocompatibility requirements of a biomaterial, as defined in international standards, which is not typical of biodegradable systems.

This chapter will describe the processing of new stiff and bioactive composites composed of a biodegradable starch-based blend reinforced with bioactive glass (Bioglass) filler as an example of the way to go on the development of new composites for bone-related applications.

8.2 MATERIALS AND METHODS

8.2.1 Materials

The study material was a biodegradable thermoplastic blend of corn starch with ethylene–vinyl alcohol (50/50 wt%), designated as SEVA-C, supplied by Novamont, Italy, with a melt flow index (MFI) value of 0.71 g/10 min (170ºC, 49 N).

As filler, a 45S5 Bioglass powder, supplied by USBiomaterials Corp., was used with a composition of 45 SiO_2, 24.5 CaO, 24.5 Na_2O, and 6.0 P_2O_5 in wt%. The powder used exhibited one granulometric distribution between 90 and 53 μm, as determined by laser scattering analysis using a model Coulter LS 100 particle size analyzer. The compound will be referred to as 10-SEVA-C/Bioglass.

8.2.2 Extrusion Compounding

The compounds were produced in a Leistriz AG-LSM 36/25D modular co-rotating twin-screw extruder (TSE). The configuration of the screw was designed to promote the best possible interaction between the filler and the polymer and to minimize any eventual thermal degradation of the starch-based blend arising from the high shear rates imposed to the melt. The presence of Bioglass particles increases the viscosity of the melt, leading to high viscous heat dissipation that requires special caution to minimize thermal degradation of the highly thermo-sensible matrix. A screw speed of 40 to 60 rpm and a temperature profile (from feeding to die zone) of 150 to 175ºC were used during compounding. The cooling of the extrudate was performed in air. No quenching was applied.

The SEVA-C/Bioglass composites were injection molded from these compounds, under optimized conditions, in a Klockner-Ferromatik Desma FM20 machine into small dumbbell ASTM tensile samples with a rectangular cross section (2×4 mm^2).

8.2.3 Mechanical Testing

The composites were tensile tested in an Instron 4505 machine, using a resistive extensometer, in order to determine the secant modulus at 1% strain ($E_{1\%}$), the ultimate tensile strength (UTS), and the strain at break (ε_r%). The tests were conducted in a controlled environment (23ºC and 55% relative humidity [RH]) using a crosshead speed of 5 mm/min (8.3×10^{-5} m/s) until 1% strain and then increased to 50 mm/min (8.3×10^{-5} m/s) until fracture. The fracture surfaces were examined by scanning electron microscopy (SEM) in a Leica Cambridge S360 microscope.

8.2.4 Soaking in Simulated Body Fluid (SBF)

Standard *in vitro* bioactivity tests were carried out to evaluate the formation *per se* (or not) of an apatite layer on the surface of the composites, considered an indicator of the *in vivo* bioactivity of the composite.

The SEVA-C samples and SEVA-C composites were soaked in a simulated body fluid (SBF) at 37ºC and pH = 7.35 for several periods of time up to 30 days. The SBF has a composition similar to human blood plasma (Table 8.1) and has been extensively used for *in vitro* bioactivity tests.[72] At the end of each immersion period, the samples were rinsed with distilled water and dried in a controlled environment (23ºC and 55% RH).

8.2.5 Morphological and Elemental Analysis

The surface morphology and the corresponding calcium/phosphorus (Ca/P) ratios of any film detected were analyzed (before and after immersion in SBF) using scanning electron microscopy and energy dispersive spectroscopy (SEM/EDS). The calcium/phosphorus ratio (Ca/P) of the films

TABLE 8.1
Ion Concentration (m*M*) of SBF and Human Blood Plasma

	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
SBF	142.0	5.0	2.5	1.5	147.8	4.2	1.0	0.5
Human plasma	142.0	5.0	2.5	1.5	103.0	27.0	1.0	0.5

Data from Ogino, M., Ohuchi, F., and Hench, L.L., *J. Biomed. Mater. Res.*, 14, 55, 1980.

TABLE 8.2
Tensile Properties of Conventionally Injection Molded SEVA-C and SEVA-C–Bioglass Composites

Filler (wt%)	Granulometric Distributions[a] (μm)	$E_{1\%}$[b] (GPa)	UTS[c] (MPa)	ε_r[d] (%)
0	—	1.86 ± 0.12	42.3 ± 2.7	14.7 ± 6.5
10% Bioglass	90–53	3.71 ± 0.24	45.7 ± 3.0	1.9 ± 0.2
10% HA	≅ 7	3.47 ± 0.15	47.5 ± 3.4	1.9 ± 0.2

[a] 90% of the particles (below this size).
[b] $E_{1\%}$ secant modulus at 1% strain.
[c] UTS ultimate tensile strength.
[d] ε_r strain at break.

was determined using well-stabilized subroutines for EDS semiquantitative analysis. Thin-film x-ray diffraction (TF-XRD) (incidence angle of 1°) was used to characterize the crystalline/amorphous nature of the films and to identify any crystalline phases present after immersion in SBF (results were compared to nonimmersed controls).

8.2.6 Solution Analysis

The solutions of the bioactivity tests were analyzed by induced coupled plasma emission (ICP) spectroscopy in order to determine the evolution of the elemental concentration of Ca, P, Si, and Na ions as a function of immersion time.

8.3 RESULTS

8.3.1 Mechanical Results

Table 8.2 shows the tensile test results for SEVA-C–Bioglass composites and SEVA-C/HA (included as reference for comparison purposes). This data refers to specimens obtained under optimized processing conditions.

As expected, and as already reported in previous works,[63,64] the addition of stiff fillers (such as HA or other types of bioactive glasses) to a starch-based blend improves significantly the respective modulus. The 10-SEVA-C–Bioglass composite presents slightly higher values of stiffness and strength than the HA-filled materials. Nevertheless, the HA particles present a much smaller average particle size and a much higher composite dispersion compared with the Bioglass particles. The mechanical properties obtained with this large-particle reinforcement (a modulus of 3.7 GPa and a UTS of 45.7 MPa) are better than those obtained for an HDPE-based matrix.[30]

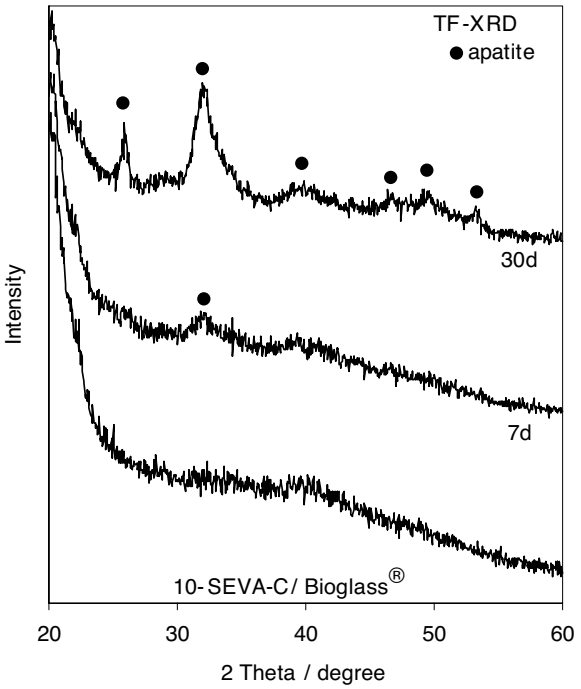

FIGURE 8.1 TF-XRD patterns of the surfaces of 10-SEVA-C–Bioglass after soaking in SBF for several periods.

8.3.2 Soaking in SBF

TF-XRD patterns of the surface of SEVA-C composites after soaking in SBF for several periods (using nonimmersed samples as controls) show several diffraction maxima that can be assigned to an apatite-like phase (ASTM JCPDS 9-432), as can be seen in Figure 8.1. The intensity of the apatite peak increases with longer soaking times in SBF. Furthermore, a larger number of reflections are noticed for longer times, which corresponds to the growth of an apatite-like layer at the composite surface. The partial amorphous nature of the formed apatite layer resembles the typical human bone apatite.

Figure 8.2 and Figure 8.3 show the SEM images and EDS spectra of the SEVA-C + 10% 45S5 surface before and after soaking in SBF for different periods. It can be seen in Figure 8.2 that after the immersion in SBF for 7 days, a dense and uniform apatite layer is deposited at the surface of SEVA-C composite.

As the soaking time increases, it can be seen that the film becomes more compact and dense. Moreover, at higher magnifications, a finer structure where needlelike crystals are agglomerated together is evident (see Figure 8.2c).

EDS spectra (Figure 8.3) shows a strong signal ascribed to P and Ca after soaking in SBF.

As the soaking time in SBF increases, the intensity of Ca and P signals is significantly increased. Furthermore, the films formed on SEVA-C composites have Ca/P ratios in the 1.5–1.8 range, i.e., between tricalcium phosphate (TCP) and hydroxyapatite, which is further confirmation of the apatite-like nature of the film formed. These values were determined using well-stabilized subroutines for EDS semiquantitative analysis.

The ICP measurements of Ca, P, Si, and Na concentrations are presented in Figure 8.4 as a function of immersion time in SBF solution. Some differences can be observed between the SEVA-C samples and SEVA-C + 10 wt% 45S5 composites. For SEVA-C composites, after 7 days of

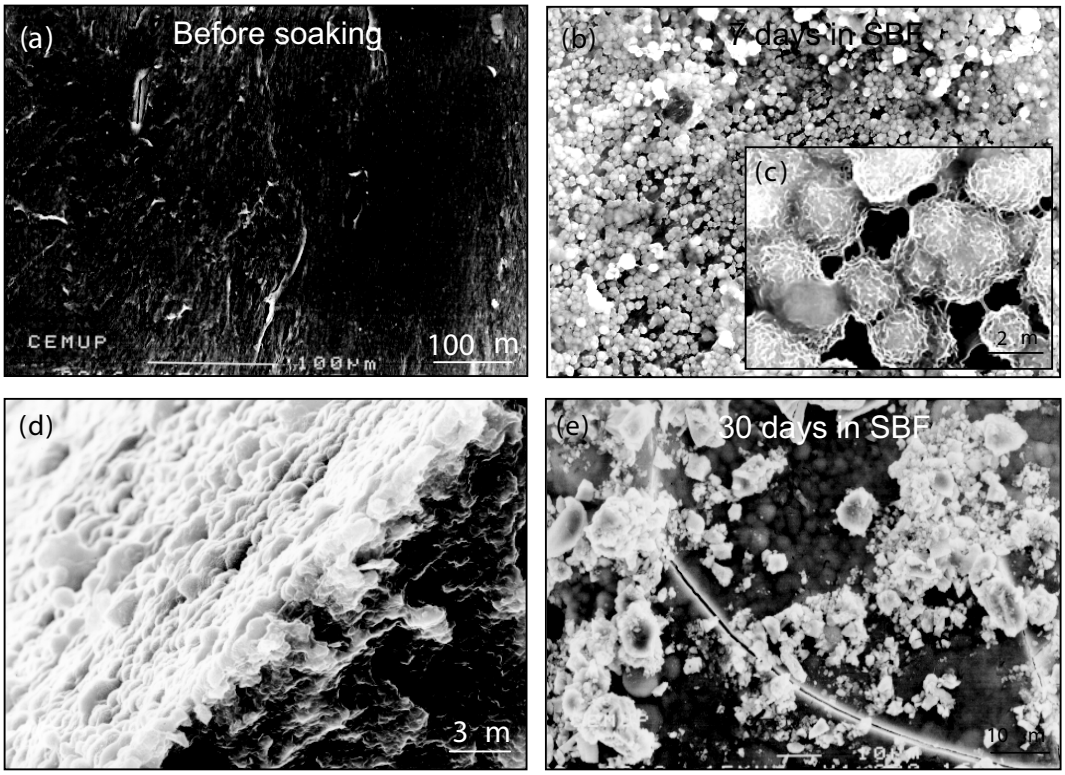

FIGURE 8.2 SEM micrographs of the surface of 10-SEVA-C–Bioglass after soaking in SBF for (a) 0, (b) 7, and (e) 30 days; (c) magnification showing a detail of the structure presented in (b); (d) cross section.

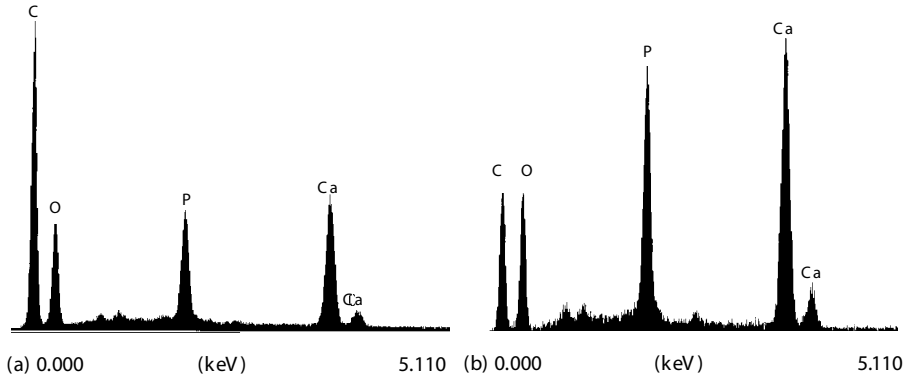

FIGURE 8.3 EDS spectra of the surface of 10-SEVA-C–Bioglass after (a) 7 and (b) 30 days of immersion in SBF at 37ºC.

soaking, an increase in the amount of Si in the SBF can be noticed, as well as a slight increase of Na concentration, which is attributed to the dissolution of the bioactive glass particles (as shown in Figure 8.4a).

This dissolution provides favorable sites for the formation of CaP nuclei during the initial period of immersion. Then, as the immersion time increases, Ca and P concentrations decrease gradually, probably due to the apatite formation caused by the consumption of the calcium and phosphate ions in SBF.

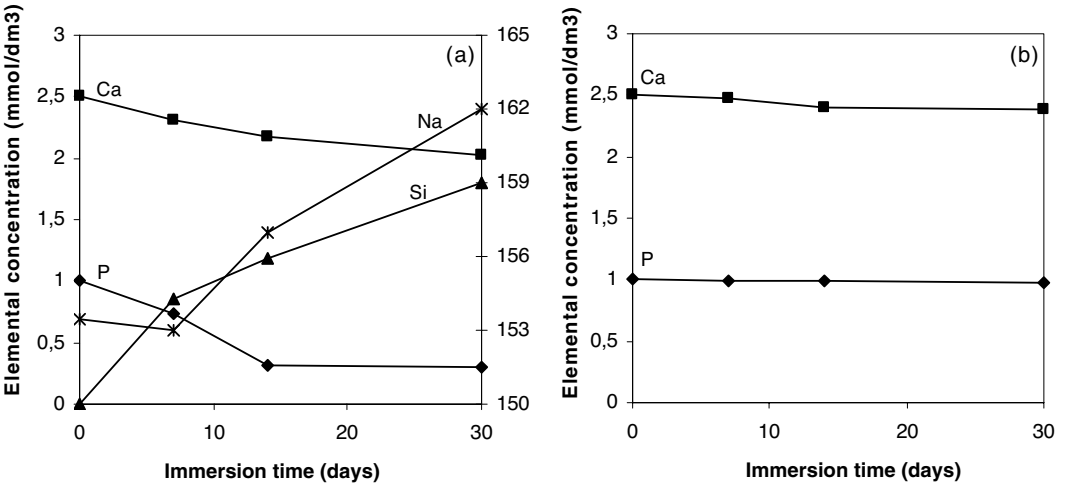

FIGURE 8.4 Evolution of Ca, P, Si, and Na concentrations in the SBF solution as a function of the immersion time for (a) 10-SEVA-C–Bioglass and for (b) SEVA-C.

In the case of SEVA-C, no change was observed in the Ca and P concentrations (as can be observed in Figure 8.4b). As already reported in previous papers,[66,73] SEVA-C shows a nonbioactive behavior when immersed in SBF solution.

8.4 DISCUSSION

The merit of composite materials is that by controlling the volume fractions and local and global arrangement of the reinforcement phase, the properties and design of an implant can be varied and tailored to suit the mechanical and physiological conditions of the host tissues. In this respect, polymeric matrix composite biomaterials are particularly attractive as their mechanical performance can be modified by means of an adequate control of the manufacturing process, which is essential to guarantee a bone equivalent mechanical behavior. However, the integration of the implant into the surrounding bone due to the establishment of an enduring interface between implant and living bone is also a crucial factor to its success. Hence, the implants should be both surface compatible and mechanically compatible with the host tissues. In this perspective, composite materials offer a greater potential of structural biocompatibility than homogenous monolithic materials.[19,20]

As described by Bonfield,[26] composite materials can mimic more efficiently the structure and properties of bone and offer a great potential to solve the problems associated to interfacial stability and stress shielding. This is due to the fact that the modulus and fracture toughness of a composite is a function of many variables that can be controlled by adequate composite formulation and its respective processing, such as the modulus of the matrix material, as well as the modulus, the volume fraction, the aspect ratio, and the orientation of the reinforcing phase.[26]

In our case, the final mechanical performance of particulate filler composite depends on the granulometry of the filler, its volume amount, and the respective processing conditions. In general, the incorporation of ceramic filler into the polymeric matrices leads to higher modulus, while the UTS and the strain at break are usually decreased. This behavior is typical of particulate-filled polymeric matrix composites and is in agreement with other studies for ceramic-filled and glass particle–filled composites.[63,64] For composites based on 45S5 Bioglass with a granulometric distribution between 53 and 38 μm, the increase in the filler amount leads to a decrease of the respective tensile strength, which is associated with the very low aspect ratio of the particles and the poor interfacial interaction between the filler and the matrix, which limits the load transfer during the

mechanical loading of the composite.[5] The observation of the tensile fracture surfaces of composites of SEVA-C with 10-wt% 45S5 suggests a poor interfacial interaction between the filler and the matrix, which is in agreement with the relatively low values of strength obtained for such composites.

One way of improving the interfacial interaction between the composite phases can be by the use of chemical-coupling methodologies that result in a higher degree of filler–matrix interaction and improved mechanical performance.[6,7] This type of approach should be conducted in future studies in order to develop a more cohesive interface between the matrix and the reinforcement phases. Nevertheless, the obtained tensile test results and the tensile fracture surfaces of these composites indicate that the combined use of TSE with optimized routines in injection molding might allow for the production of biodegradable composites exhibiting a homogeneous distribution of the filler and attractive values of stiffness and strength.

The bioactivity tests showed that the Bioglass–SEVA-C composite developed an apatite layer at its surface after immersion in a SBF solution, which confirms the bioactive character of the composite (see Figure 8.1 and Figure 8.2) and its expected *in vivo* bone-bonding behavior. Furthermore, these results show that the Bioglass filler is highly bioactive, since for an amount of only 10 wt%, it is possible to obtain an apatite layer after 7 days of immersion. Of course, this is only possible due to the water-uptake ability of SEVA-C, which will be explained shortly. In fact, for SEVA-C–HA composites, an amount of at least 30-wt% HA is required to observe the same type of behavior.[62,74] As stated before, the reason for the observed bioactivity is the higher reactivity of the Bioglass filler together with the water-uptake ability of the polymer, which also allows the inner Bioglass particles to play an active role. In fact, the high water-uptake capability of the matrix favors the dissolution of the bioactive glass particles, leading to an increase of Si and Na concentration.

As proposed by Hench,[75–77] the first reaction of this type of bioactive glass surface is ion exchange, in which Ca^{2+} and Na^{+} in the glass exchange for H^{+} in the solutions, resulting in an increase in pH of the solution as well as in the formation of a hydrated silica gel layer. It is also reported that the hydrated silica layer on the surface of Bioglass provides favorable sites for the calcium phosphate nucleation.[75,78,79] During the growth of the CaP nuclei, the consumption of the Ca and P ions from the surrounding fluid occurs, resulting in the observed decrease of the Ca and P concentration in the solution. The growth of the CaP nuclei leads to the formation of an apatite layer at the surface of the SEVA-C–Bioglass composite. This is supported by ICP measurements as shown in Figure 8.4. It can be concluded that a weight amount of 10% is enough to confer a bioactive character to SEVA-C–Bioglass composites.

8.5 CONCLUSIONS

Stiff and bioactive composites based on a biodegradable starch-based blend and a bioactive glass (Bioglass) filler were successfully produced. The SEVA-C–Bioglass composites are clearly bioactive as shown in *in vitro* bioactivity assays and combine interesting values of stiffness and strength coupled with a degradation behavior under simulated physiological solutions. The *in vitro* formation of an apatite layer at the surface of the composites is an indication of the bone-bonding ability of the developed materials. The obtained results suggest the high potential of these types of composites for a wide range of applications in the field of bone defect filling applications.

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9 Mechanical Characterization of Biomaterials

J. F. Mano, N. M. Neves, and Rui L. Reis

CONTENTS

9.1 INTRODUCTION

Especially for implanted materials that will withstand mechanical stresses in clinical use (e.g., in vascular or orthopedic applications), a proper mechanical characterization is among the most important physical tests that must be carried out. This will give the viability of applying the material in terms of its geometrical integrity, both at short term (determined by the elastic modulus and strength of the specimens) and long term (obtained from extrapolated creep/stress relaxation tests or fatigue). Before any specific mechanical test, one needs to specify clearly the functionality of the biomaterial, how it will work, the tissues with which it will be in contact, and the kind of mechanical environment that it will be facing. This will determine, for example, if it is most convenient to test the material in tension, compression, bending, shear, or in another more specific mechanical configuration. If the material swells easily in aqueous solutions, and if it is thermally sensitive near room temperature, one should test it while immersed in physiological simulated solutions and ideally at 37ºC. More complex tests are needed if one tries to simulate the mechanical environment of the implant; an example could be the evaluation of the implant–bone interface in orthopedic or dental applications, to evaluate the efficiency of coatings or cements as attachment tools.

Besides the mechanical properties of the implanted material, it is also important to know the mechanical behavior of the tissues that it will contact; generally, one should choose a material that will behave similarly to it. An orthopedic implant should have enough stiffness and strength to support the stresses developed in bone; however, too-stiff materials may transfer inefficiently the loads into bone (stress shielding), inducing bone resorption. In cardiovascular or skin applications, one should have materials that are relatively compliant at low strains but with high strengths, as it

TABLE 9.1
Mechanical Properties of Different Biological Materials

Biological Material	Tensile Modulus/GPa	Strength/MPa
Insect cuticle	6–10	80
Whale bulla	30	33
Antler	7.7	179
Cortical bone (longit. direct.)	17.7	133
Cortical bone (transv. direct.)	12.8	52
Cancellous bone	0.4	7.4
Dentine	11–12	25.0–39.3
Enamel	45–85	10–76
Articular cartilage	10.5×10^{-3}	27.5
Fibrocartilage	0.159	10.4
Ligament	0.303	29.5
Tendon	0.401	46.5
Skin	$0.1–0.2 \times 10^{-3}$	7.6
Arterial tissue (longit. direct.)		0.1
Arterial tissue (transv. direct.)		1.1
Intraocular lens	5.6	2.3

Data from Calvert, P., *MRS Bull.*, 17, 37, 1992; Ramakrishna, S. et al., *Comp. Sci. Technol.*, 61, 1189, 2001.

happens with the corresponding biological materials, in order to integrate well with the living tissues. Table 9.1 summarizes some mechanical properties of biological tissues. We may conclude that the mechanical parameters of tissues cover a large spectrum of values. It is also known that cells respond to mechanical stresses (mechanotransduction); if they are resident in porous biomaterials, e.g., for tissue engineering purposes, the stresses felt by the cells will be determined by the mechanical properties of the scaffold itself.[1]

This chapter intends to provide introductory fundaments on general mechanical characterization techniques that may be useful in the context of the development of materials for biomedical applications. We will concentrate on the measurement of the mechanical and solid-state rheological macroscopic properties of materials. Out of this scope are the techniques that allow measurements in specific points in the sample, such as micro/nano-indentation, which are useful for heterogeneous systems. A good example is the use of atomic force microscopy for the determination of the microelastic properties of biological materials.[4]

9.2 QUASI-STATIC TESTS

The effect of mechanical stress over biomaterials has been always a concern in the design of new materials and processing methods. Quasi-static tests are performed in universal testing equipments in which a prescribed displacement rate is selected and controlled.

The universal tensile testing equipment consists of a rigid frame and a moving rigid arm. The movement of the rigid arm is transmitted through the specimen into an instrumented load cell. The load cell enables the recording of the evolution of the load produced by the arm displacement during the test. The resulting load will be affected by the load dissipated by the deformation of the test specimen. Thus, a perfectly elastic material should lead to a constant variation of the load with a constant displacement of the rigid arm. The degree of deviation of this linear behavior is an indication of the viscoelasticity character of the materials being tested.

The modes of operation and the mountings can be selected to perform tensile tests if the testing specimen is pulled or compression tests if the test specimen is pressed against the load cell. Similarly, it can be flexured when the supports of the test specimen are not aligned with the center of gravity of the specimen.

The choice of the type of test to be performed depends to a great extent on the mechanical environment to which the material will be submitted in service. That stress environment should be representatively modeled in the test so that reliable conclusions can be drawn. If a bone graft is to be studied, it is probably more relevant to study the compression properties than the tensile properties. Often, the structure of the material to be studied determines the type of test to be used. For example, a foamed porous specimen will be much more difficult to study in tensile tests than in compression, mostly because of difficulties in clamping.

In order to obtain reliable data from quasi-static tests, a number of requirements should be accomplished. The geometry of the testing specimens is a very important parameter. The dog bone tensile bar (e.g., ISO 527-1, ASTM D638-02a) is designed so that it is easy to clamp, and it concentrates most of the strain in the linear region of the specimen, enabling extraction of reliable engineering mechanical properties from the testing curve. Whenever more accurate results are required, it is advisable to use a suitable extensometer (strain transducer), enabling one to follow the exact amount of strain that is produced in the representative region of the test specimen (the central linear region). It is very important to use homogeneous testing specimens to avoid any stress concentration effects either from the geometry or from inadequate production of the test specimen.

If the materials have anisotropy of mechanical properties (as in fiber-reinforced composites), it might be of interest to produce different testing specimens (e.g., injection-molded plates) from which the tensile test specimens can be cut in the directions of interest, capturing the anisotropy in mechanical properties.

The result of a quasi-static test is a curve of the evolution of the load with the displacement. From that information and from the geometry of the testing specimen, it is possible to derive properties such as the elastic modulus, the stress and strain at yield (transition elastic/plastic-dominated deformation), and the stress and strain at break. Those engineering properties are frequently the goal of a mechanical testing study.

Polymeric materials display viscoelastic behavior. This is visible in quasi-static tensile tests because there is no linear region of the stress–strain curve. The viscoelastic character of polymeric materials has important implications in its mechanical performance, being highly dependent on the deformation rate and on the testing temperature. Relationships between temperature and strain rate (superposition temperature–time) can be derived, which will be discussed further in another section of this chapter.

It is important to highlight that the tensile modulus can be calculated in many different ways, depending on the particular interest of the user. The elastic modulus is one particular way of characterizing the stiffness; the calculation uses values taken from the steepest positive region of the stress–strain curve. Thus, the elastic modulus defines the maximum value of the stress–strain relationship along the curve. It is also very common to use a secant modulus at a predefined strain level. Thus, it is very important to specify clearly what tensile modulus is in use and the exact calculation procedure followed, enabling comparison of data from different sources.

One issue to be addressed in the experimental planning is the sample size. The standards regulating the quasi-static testing methods (ISO, ASTM) provide general guidelines to define a suitable number of specimens to be tested. It is well known that the sample size, being a statistics requirement, should be adjusted to the degree of accuracy needed in the results and should take into account the scatter in the results obtained. However, it should be noted that the standards are not always well adapted to the constraints faced in research, and it is advisable to use larger sample sizes in research studies.

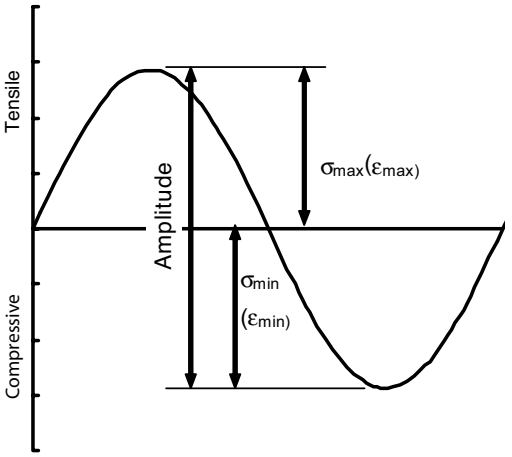

FIGURE 9.1 A tensile-compressive fatigue test wave, illustrating the amplitude and maximum and minimum values of the control parameter stress (or strain).

9.3 FATIGUE

Fatigue tests are cyclic dynamical mechanical tests in which a cyclic stress (or strain) is applied to the test specimen and the strain (or stress) response of the material is recorded.

The maximum stress (or strain) can be defined as the largest stress (or strain) applied to the test specimen during the cycle. The minimum stress (or strain), conversely, is the minimum value applied during the cycle. A tensile/compression fatigue test will have a positive maximum stress (or strain) and a negative minimum stress (or strain), as shown in Figure 9.1.

The mean stress is the algebraic midpoint between the maximum and the minimum. The mean stress can be positive, negative, or zero. When it is nonzero, this test can be modeled as a creep experiment (constant stress) combined with a zero-mean-stress (or strain) fatigue experiment (Figure 9.2). Failure by a creep-related fracture process is of primary importance in engineering polymers. Such tests are also known as static fatigue experiments.

Both the amplitude of the oscillation and the shape of the wave (triangle, sinusoidal, square) control the amount of energy applied to the material in each cycle. Another parameter to be controlled in the fatigue tests is the frequency. The frequency is often used to shorten the testing time. However, reliable high amplitudes are difficult to obtain with high frequencies. Thus, preliminary tests are required to analyze the viability of performing accurate fatigue experiments with the desired set of parameters.

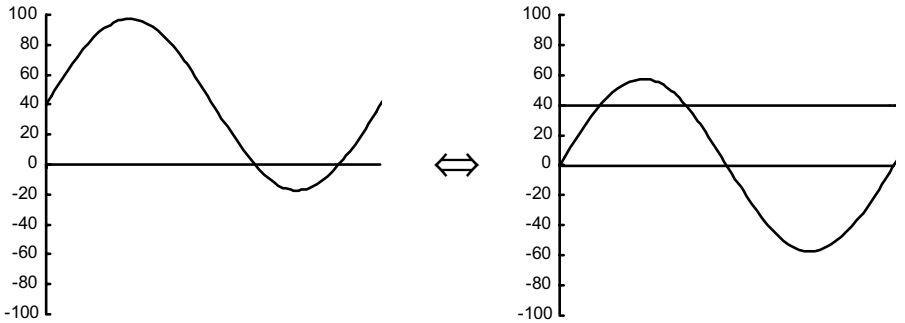

FIGURE 9.2 Illustration of the equivalence between a nonzero-mean fatigue test and a creep experiment superimposed to a zero-mean fatigue test.

Viscoelasticity limits the testing parameters in the design of fatigue experiments. Tensile/compression fatigue experiments are difficult to perform in materials in which pronounced viscoelasticity is present, because of the possibility of buckling after a number of cycles. Thus, those materials are frequently studied in tensile fatigue, the specimen being always subjected to tensile stresses or strains (wave always positive).

Fatigue experiments can be controlled by stress or by strain. Depending on the thermal dissipation of the material being tested, the strain-controlled tests can be more stable than stress-controlled tests. Heat generated by deformation in materials with low thermal conductivity (such as polymers) can lead to softening and to a premature catastrophic failure of the material when the stress is in control. Conversely, in a strain-controlled fatigue test, an eventual softening of the material leads to lower levels of stress being produced by the softening of the material caused by mechanical heat dissipation, a thermal equilibrium being reached with the environment.

Fatigue tests used to evaluate biomaterials can be categorized as follows[5]:

1. Stress/life (S/N curves)
2. Fracture mechanics approach
3. Fatigue–wear approach using multiaxial loading and simulated physiologic environments

The first two methods are used primarily for the materials-screening process and are useful for the initial process of materials selection of implant materials, particularly joint prostheses. The third method is considered an *in vitro* evaluation to determine the fatigue performance close to a physiologic environment and is normally a precursor to animal experiments. The first two approaches are found to be less expensive. The third approach is less economical and requires special devices modeling the service conditions.

The S/N approach is normally done using smooth specimens in a physiologic environment. The advantage of this approach is that it represents both initiation and propagation of cracks in the aggressive environment.

In the fracture mechanics approach, the fatigue–crack propagation of the biomaterials are studied by (1) long cracks (> 3 mm) using compact-tension specimens or (2) small cracks (1–250 μm) using micro-indentation methods in a servo-hydraulic machine. This approach, often done in a physiologic environment, is good for studying brittle implant materials like ceramics[6,7] and dental composites,[8,9] where sensitivity to initial flaw sizes and crack propagation rates determine the lifespan of the implant.

9.4 CREEP EXPERIMENTS

9.4.1 VISCOELASTICITY

Perfect elastic solids respond instantaneously with a deformation under a static load, maintaining the strain with time. Such systems store all the energy utilized in the deformation induced by the developed stress, which is completely used in an eventual recovery. Within the linear regime, one should have ideally $\sigma = E\varepsilon$, where E is the Young's modulus. Conversely, in perfect Newtonian liquids, the stress is proportional to the strain rate: $\sigma = \eta d\varepsilon/dt$, where η is the viscosity. In this case, all the mechanical energy used in the deformation of a perfect viscous liquid is dissipated as heat, preventing the recovery of its shape. Many materials, including most of the biological tissues (possibly excepting dental enamel and echinoderm skeletons) and polymers, feature a hybrid behavior called viscoelasticity.[10,11] Their mechanical response depends on the timescale of the experiment, and different types of experiments will now be analyzed in order to study this phenomenon. In this section, we will analyze creep tests, which are among the simplest experiments that reveal the viscoelastic nature of materials.

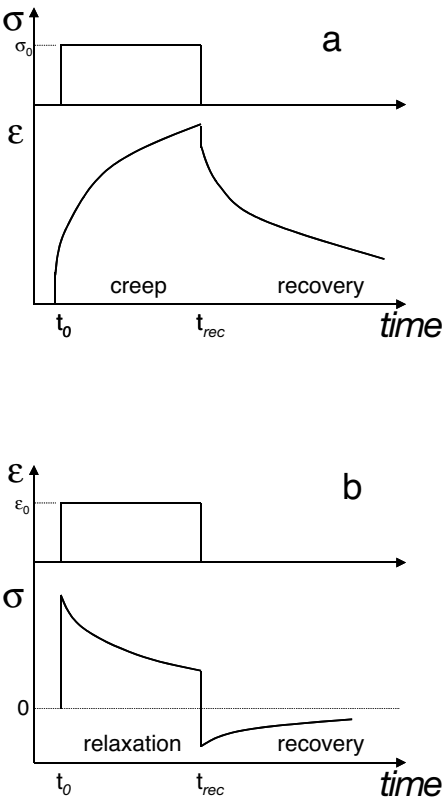

FIGURE 9.3 (a) Creep experiment protocol followed by a recovery process ($\sigma_0 = 0$); (b) stress relaxation test, where the stress level is measured under constant strain.

9.4.2 Definition

In creep experiments, a step in stress σ_0 is induced, and the strain is monitored against time, $\varepsilon(t)$. The total mechanical response of a specimen includes perfect elasticity, total irreversible flow, and a coupling of elastic and viscous components, called anelasticity (see Figure 9.3a). As $\varepsilon(t)$ depends directly on σ_0, in the linear regime one has proportionality between these two variables for the same time frame. In this case, it is convenient to define the creep compliance function that is a material property: $D(t) = \varepsilon(t)/\sigma_0$.

Creep measurements are important if a material is subjected to a series of static loads. An example is the use of polymeric filaments for sutures. Figure 9.4a shows the creep compliance of polyvinylidene fluoride monofilament sutures at different temperatures. As expected, as the temperature increases, the material presents higher deformation capability.

There are several commercially available devices that perform creep tests, and those have been widely used in the study of biomaterials. Besides the tests in synthetic systems, it is also important to know such properties for biological materials; a recent study[13] looked at the failure of Achilles tendons, where the corresponding mechanism is far to be elucidated, despite being one of the most frequently injured tendons in humans. It was found that there was no significant relationship between applied stress (within the range 35 to 75 MPa) and time to failure, but time to failure decreased exponentially with increasing initial strain (strain when target stress is first reached) and decreasing failure strain. Also, creep experiments have been performed in bone[14] or dentin.[15] In the first case, it was proposed that the long-term deformation in bone should be due to displacements occurring at the cement lines.

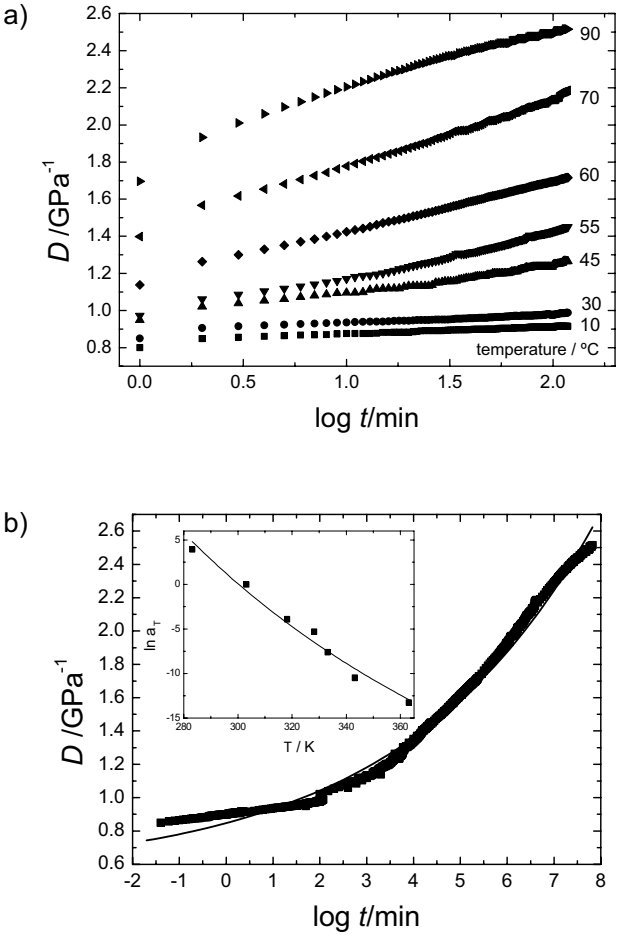

FIGURE 9.4 Top: Experimental creep on polyvinylidene fluoride monofilaments as a function of log time at different creep temperatures, between 10 and 90ºC. Bottom: Master curve at 30ºC obtained with the same data. The inset shows the temperature dependence on the shift factor, fitted with an equation derived from free-volume concepts. (Adapted from Mano, J.F. et al., *Polymer*, 44, 4293, 2003.)

9.4.3 Time–Temperature Superposition

In many cases, the viscoelastic behavior of a material at different temperatures may be related by a simple change in the timescale. Taking Figure 9.4a as an example, and fixing as a reference temperature 30ºC, it can be seen that the curve at 10ºC can be superimposed quite well with the one at 30ºC, by simply horizontally shifting to lower times by a factor log a_T, where a_T is called the shift factor. We may write, more generally, that under this time–temperature superposition, we have $D(t,T,\sigma_0) = D(t/a_T,T_{ref},\sigma_0)$. Therefore, the curves at temperatures above 30ºC may be also superimposed to the 30ºC curve by shifting each of them to higher times with a corresponding log a_T. The resulting plot with all such superpositions corresponds to the master curve of the material at 30ºC. Figure 9.4b shows this result, along with a fitting using a hyperbolic sine function.[12] The shift factors are plotted against temperature in the inset of Figure 9.4b. Such kind of data may be adjusted to different models, which may give further insights about the mechanisms at the molecular level assigned to the creep process. In this particular case, the results were fitted with a model based on the free-volume theory.[12] Such interpolations may be used to shift the master curve to any desired temperature. Therefore, information about $D(t)$ in a certain temper-

ature may be transformed into another temperature just by knowing the shift factor between these two temperatures. For the particular case analyzed, one could have the creep compliance curve, for example, at 37ºC. Furthermore, the time–temperature principle allows one to predict the long-term deformation behavior of the monofilaments from short-term tests, at different temperatures. From the 120 min set of experiments shown in Figure 9.4a, one achieves information on the creep behavior at 30ºC for times longer than 190 years. Therefore, such tools may be very useful for predicting the mechanical performance of nondegradable (or low degradable) implants for times that are not easily experimentally accessible. Another example is the use of the time–temperature superposition in the prediction of the creep behavior of bone cement.[16] As creep of acrylic bone cement may contribute to loosening of cemented total joint replacements, such studies may help us better understand the time-dependent mechanical properties of these systems and help us find ways to reduce the sensitivity to creep. In that study, an analysis was also done on the effect of structural relaxation on the creep behavior; this reversible phenomenon occurs when materials are kept below their glass transition temperature, T_g (as is the case with such acrylic systems in use). In this region, the physical properties slowly change with time up to equilibrium values[17]; this process is termed *physical aging*. This is particularly important if implanted biodegradable materials have T_g close but above physiological temperature. Poly(lactic acids) are among the materials in such conditions (e.g., poly[*L*-lactic acid] [PLLA] has a T_g around 65ºC). Another study in the same line analyzed the influence of the injection time of the material on the creep behavior of the bone cement.[18] It was found that delayed injection time could considerably increase creep.

9.4.4 The Boltzmann Superposition Principle

The basis of this principle is that in a multistress occurrence, each loading step makes an independent contribution to the final deformation, which is obtained additively from all contributions. If changes of stress occur at different times, τ_1, τ_2, …, then

$$\varepsilon(t) = \sigma_0 D(t) + \Delta\sigma_1 D(t-\tau_1) + \Delta\sigma_2 D(t-\tau_2) + \ldots \tag{9.1}$$

where $\Delta\sigma_i$ are the step changes of stress at time τ_i (i.e., $\Delta\sigma_i = \sigma_i - \sigma_{i-1}$). The Boltzmann superposition principle states that the response of a material to a given load is independent of the material response to any previous load histories. Equation 9.1 may also be used to model the recovery process that corresponds to the removal of the creep stress at a certain time t_{rec} (see Figure 9.3a); in this case, one should have, at a given time $t > t_{rec}$, $\varepsilon(t) = \sigma_0 D(t) - \sigma_0 D(t - t_{rec})$. Considering that at $t = 0$ the specimen is not affected by its previous stress history, then for more continuous and complex stress variation, one may generalize Equation 9.1 to

$$\varepsilon(t) = \int_0^t D(t-\tau)\frac{d\sigma(\tau)}{d\tau}d\tau \tag{9.2}$$

The Boltzmann superposition principle may be useful if the intent is to follow the deformation of a given implant that is subjected to different loads during a period of time — for example, a rest during 7 hours followed by standing during 2 hours, sitting during 3 hours, again standing during 2 hours, and running for 1 hour. If $D(t)$ of the material implanted is known and if the stresses that are developed in each situation are also known, it will be possible to predict the final geometrical status of the implant.

9.5 STRESS RELAXATION EXPERIMENTS

In a stress relaxation experiment, a constant strain ε_0 is imposed at $t = 0$ and held constant. Under such conditions, viscoelastic materials display a decrease of stress with time, $\sigma(t)$ (see Figure 9.3b). In this case, the relevant material property is the relaxation modulus $E(t) = \sigma(t)/\varepsilon_0$. For linear viscoelastic materials, $E(t)$ is independent of the strain level. We must be aware that most biological materials possess highly nonlinear properties. For example, it was found that stress relaxation proceeds more rapidly than creep in medial collateral ligaments, a fact that was found to be consistent with nonlinear theory.[19] Another study on mesenchymal gap tissue also described this nonlinear behavior, and such studies may be important in the understanding of osteogenesis.[20]

Note that both creep and relaxation may occur in shear and volumetric (bulk) deformation conditions; in this case, the corresponding compliance and relaxation modulus functions are $J(t)$ and $G(t)$ for shear and $B(t)$ and $K(t)$ for volumetric deformations, respectively. As for creep, both temperature/time and Boltzmann corresponding principles hold for stress relaxation. For the latter case, considering a continuous variation of strain, the stress level is given by

$$\sigma(t) = \int_0^t E(t-\tau)\frac{d\varepsilon(\tau)}{d\tau}d\tau \tag{9.3}$$

Stress relaxation experiments have also been used in the characterization of biomaterials. In some cases, the material is implanted in a well-confined cavity (thus its deformation is kept constant), and it is important to have information about its time-dependent stress level, which may have consequences on the mechanical performance of the surrounding tissues. An example is the study of the influence of wet/dry environment on the stress relaxation of bone cements.[21] Such studies point out the differences between the viscoelastic properties of biomaterials when tested in dry conditions (the most usual case) and in simulated physiological conditions — a situation that has been gaining interest and will be briefly discussed in Section 9.7 of the present chapter. Moreover, other phenomena may affect the time-dependent mechanical response in biomaterials; an important case is the hydrolytic scission of the polymeric chains occurring in biodegradable polymers, such as poly(lactic acids).[22] In such degradable systems, significant studies include tests in porous materials to be used in tissue engineering. Among other tests, compressive creep properties of porous poly(lactic acid) were monitored during degradation.[23] The results suggested that the degradation rate of porous foams could be engineered by varying the pore wall thickness and pore surface–volume ratio. In long-term tests of biodegradable materials, one should be able to discern in the results the pure viscoelastic behavior of the material and the degradation process. This task has not yet been efficiently accomplished; one hypothesis should be the combination of results derived from viscoelastic monitoring of materials with different molecular weights and the information on the time variation of the molecular weight of the same materials.

9.6 DYNAMIC TESTS

Tests that impose a periodic load or strain on the sample and measure its corresponding response constitute probably the most used tool to characterize the solid-state rheological properties of viscoelastic materials. They also allow one to obtain a series of material properties in a wide temperature and timescale range. On the other hand, the test mode is highly relevant in the context of biomaterial characterization. In fact, in many situations, implants and biological tissues are subjected to periodic loads (or cyclic changes of strain). Important examples are implants used in both dental or maxillofacial applications, subjected to the masticatory function; biomaterials used in the orthopedic field (substitution or repair of bone or cartilage bone cements, bone tissue engineering scaffolds, fixation plates and pins, etc.) that are exposed to the cyclic loads caused

by the patient movements; and devices used in cardiovascular applications that experiment the pulsed blood pressure. At the end of this section, some examples of applications of dynamic tests will be presented.

When a specimen is subjected to a sinusoidal load, with a rate defined by a frequency f (in cycles s^{-1}, or Hz) or an angular frequency $\omega = 2\pi f$ (in rad s^{-1}), the response (a strain), though sinusoidal, is neither exactly in phase with the developed stress (as it would be in the case of a perfectly elastic solid) nor 90º out of phase (as it would be for a perfectly viscous fluid); thus, the strain will lag behind the stress by some phase angle δ between 0 and 90º (Figure 9.5a). This delay is a result of the time necessary for molecular translational/rotational rearrangements. The sinusoidal stress, written in a complex notation, is as follows:

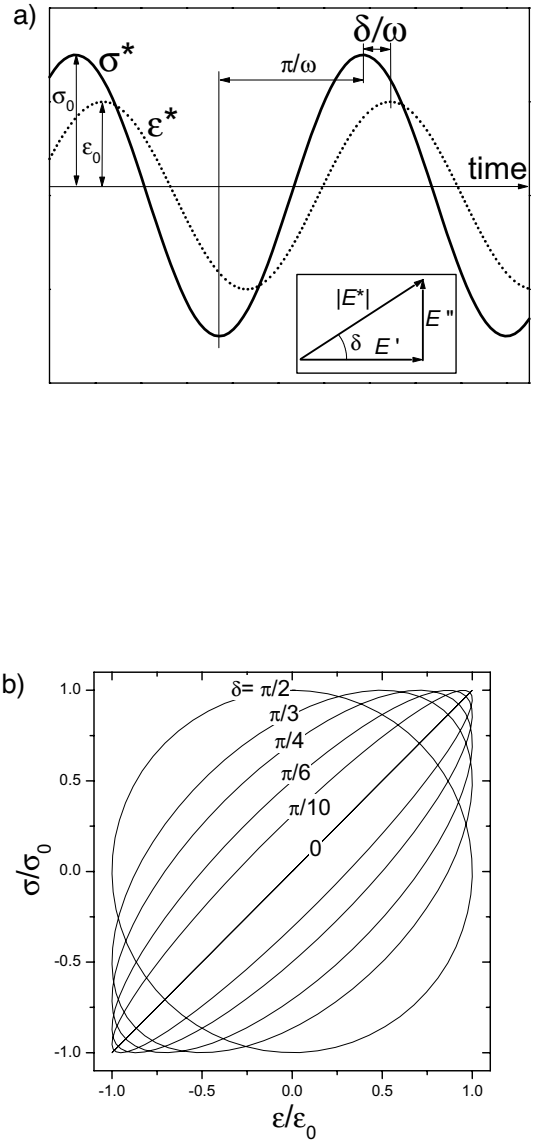

FIGURE 9.5 Scheme of the mechanical response (strain, ε) of a viscoelastic material subjected to a sinusoidal stress (σ): (a) progress of σ and ε in the time domain; (b) corresponding reduced σ-versus-ε plot, for different phase angles δ.

$$\sigma = \sigma_0 \exp(i\omega t + \delta) \tag{9.4a}$$

$$\varepsilon = \varepsilon_0 \exp(i\omega t) \tag{9.4b}$$

where σ_0 and ε_0 are the stress and strain amplitudes and $i = (-1)^{1/2}$. Note that only the response in the linear viscoelastic region will be considered. In the nonlinear regime, other harmonics can be generated, complicating the stress–strain responses. A full description within this linear region may be provided by the complex modulus, $E^*(\omega)$, or the complex compliance, $D^*(\omega)$, defined, respectively, as

$$E^*(\omega) = \frac{\sigma}{\varepsilon} = (\sigma_0 / \varepsilon_0)\exp(i\delta) = (\sigma_0 / \varepsilon_0)(\cos\delta + isen\delta) = E' + iE'' \tag{9.5a}$$

$$D^*(\omega) = \frac{1}{E^*} = \frac{\sigma}{\varepsilon} = (\varepsilon_0 / \sigma_0)(\cos\delta - isen\delta) = D' - iD'' \tag{9.5b}$$

The storage modulus, E', is the elastic, or the real, component of E^*, which is in phase with σ. The storage modulus is related to the stiffness of the material. The loss modulus, E'', is the viscous (also called imaginary) component of E^*, which is $\pi/2$ out of phase in relation to σ. E'' is associated with the dissipation of energy, as heat, due to internal friction at the molecular level. Note that the same discussion could be extended for the components of the complex compliance, D^*. The dissipation of energy in a complete cycle, per unit of volume, is given by the area within the σ versus ε plot (Figure 9.5b):

$$E_{dis} = \oint \sigma d\varepsilon = \pi E'' \varepsilon_0^2 = \pi D'' \sigma_0^2 \tag{9.6}$$

The maximum stored elastic energy is given by

$$E_{st,\max} = \int_0^{\varepsilon_0} E'\varepsilon d\varepsilon = E'\varepsilon_0^2 / 2 = D'\sigma_0^2 / 2 \tag{9.7}$$

The loss factor, $\tan\delta$, is E''/E' and $|E^*|^2 = E'^2 + E''^2$ (inset in Figure 9.5a). The ratio of $E_{dis}/E_{st,\max} = 2\pi \tan\delta$ is often a measure of the damping capability of a material and is called the specific loss or specific damping capacity. Biological tissues usually have excellent damping capabilities (with the possible exception of dental enamel and echinoderm skeletons). This is highly efficient in dissipating external loads caused by day life. As a classic example, movements such as walking or running make heel-strike shock waves travel through the body, which are dissipated mainly by the skeletal system (bone has quite relevant damping properties[14,15]); deficient energy dissipation can cause maladies such as osteoarthritis, stress fracture, tendonitis, migraine, back pain, or inner ear disturbance. It is therefore fundamental to design implant materials that match the viscoelastic properties of the host tissues.

In dynamic tests, the greatest changes in viscoelastic variables occur when relaxation processes happen. This is evidenced by changes in the level of E' or D' and by peaks in the corresponding imaginary components or in $\tan\delta$ in the log frequency or temperature axis. Such relaxation processes are associated with the occurrence in such axes of specific rotational or translational

molecular motions. From a phenomenological viewpoint, a relaxation process may be characterized by a distribution of characteristic times, with a given dependence on temperature. Such properties may be identified by treating dynamic results with different models. More details about the interpretation of relaxation phenomena may be found elsewhere.[10,11] However, due to the importance of this point, a brief description of the kind of molecular motions that may be found in anelastic processes in solids should be provided. In the context of biomaterials, such studies allow one to obtain a better perception about the relationship between structure and properties; for example, it allows one to clearly identify the glass transition phenomenon and other molecular mobility processes that may alter significantly the viscoelastic behavior of the material in a given temperature or frequency range, thus conditioning its general performance.

The local motions assigned to low amplitude displacements of chain segments or within the lateral groups (β, γ, δ, … relaxations in amorphous polymers) are thermally activated processes usually detected at lower temperatures (or high frequencies) than the glass transition temperature.[10] In many situations, where probably the most cited example is polycarbonate, such secondary relaxations may have important implications for the mechanical properties at room temperature, including being responsible for the high impact performance of the material. In fact, if at low temperatures the corresponding characteristic times are on the order of seconds, then at room (or body) temperature such times may be on the order of milliseconds or microseconds; this means that such relaxations are able to dissipate mechanical energy coming from very high frequency inputs or from very short time shocks. It may thus be important to study secondary relaxations if one intends to develop a biomaterial with good impact properties. It should be noted in this context that some biological materials have very good impact properties. Bone is a very good example, which can be related to the high damping properties at high frequencies (tan δ 0.08 for $f = 10^7$ Hz[25]).

The molecular motion associated with the glass transition (α relaxation in amorphous polymers) has a cooperative character, and its occurrence is accompanied by drastic changes in the mechanical properties of the material, especially if it is an unfilled amorphous one.[17] Therefore, the characterization of such process in amorphous or low-crystallinity materials is fundamental to understand their thermal properties and predict their mechanical performance.

Isolated relaxations are characterized by an E'' peak at lower temperatures (higher frequencies) than the D'' peak, with the tan δ peak situated between them. Figure 9.6 shows the general trend in terms of position and shape of the more important viscoelastic variables for a process characterized by a Dirac distribution of characteristic times. The enlargement of the peaks would increase with the broadening of the distribution of relaxation/retardation times. As a practical and representative

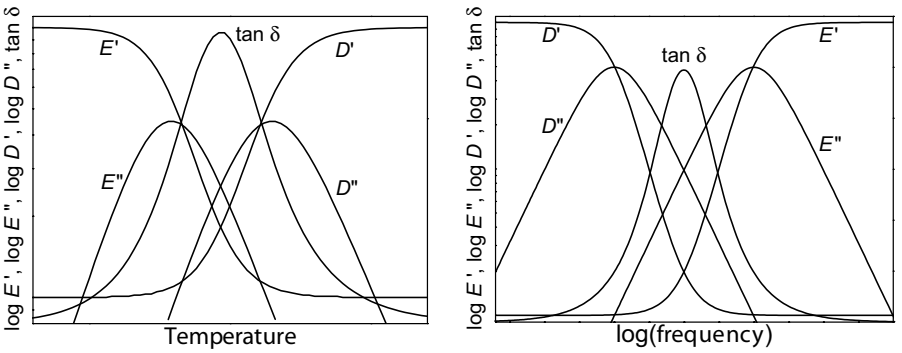

FIGURE 9.6 General relative location and shape of the most important viscoelastic parameters on both temperature and frequency axes. The data was generated assuming a relaxation with a single characteristic time, with an Arrhenius dependence with temperature.

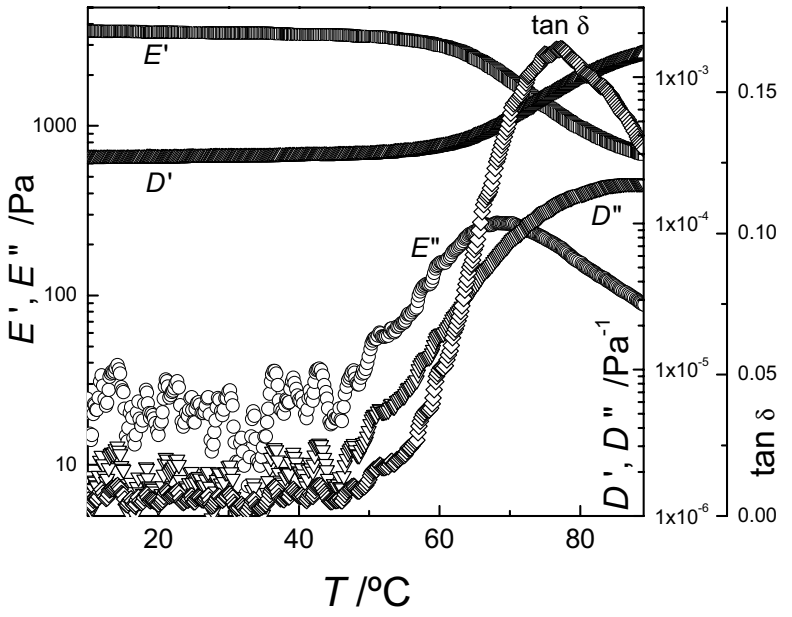

FIGURE 9.7 Mechanical spectrum at 1 Hz of a semicrystalline poly(*L*-lactic acid), studied under three-point bending. The experiments were carried out in a Perkin-Elmer DMA7 apparatus at 3ºC min^{-1}.

example, Figure 9.7 shows the variables mentioned before for the case of a poly(*L*-lactic acid) studied in a bending mode and tested at 1 Hz. It may be concluded that the stiffness of the material is unchanged up to 50ºC, with a relatively low damping (almost elastic material). Above that temperature, the occurrence of the glass transition is visible, with changes observed in all variables. The glass transition may be calculated as the temperature of maximum E''; although widely used, the temperature of the maximum of tan δ may give erroneous indications as, besides the glass transition phenomenon, the location of this peak depends on the elastomeric plateau above T_g.

Each relaxation process has its own location in an *f*-versus-*T* plot. Usually this correspondence follows a simple Arrhenius relationship for secondary processes; in those cases, the activation energy of the process can be readily obtained, together with the preexponential factor. More complicated models are used to describe the kinetics of the α relaxation,[11,17] where, above T_g, its apparent activation energy increases with decreasing temperature, due to the enhancement of cooperativity (together with the decrease of the free volume) associated with the mechanisms involved.

The instruments are limited in terms of the available frequency range, usually between 0.01 and 100 Hz. The timescale ranges may be extended by exploiting the time–temperature superposition principle, mentioned in Section 9.4.3. Frequency-scanning experiments are carried out at different temperatures, and the obtained data are shifted along the log *f* axis in order to construct master curves, which allow one to estimate behavior outside the range of the instrument.

A review of the use of such dynamic mechanical analysis techniques on different biomaterials can be found elsewhere.[24] This includes the study of biodegradable or inert materials for different applications: hydrogels and elastomers, curable systems for dental applications and bone cements, and systems for hard tissue replacement. Just some examples covering such three cases should be given herein to reinforce the importance of these types of tests.

The intercellular matrix of the disc of the temporomandibular joint consists mostly of type I collagen and proteoglycans that provide resistance to various loads. In a recent study, bovine discs were tested with different proteoglycan contents, and it was concluded that such components play an important role in determining the viscoelastic properties of the disc, enhancing the capability for distributing and reducing stresses.[26] These results may be relevant in the development of

prostheses with higher damping capability. In the context of hydrated systems, composite ligament prostheses were also developed, based on a hydrogel–polymer matrix reinforced with poly(ethylene terephthalate) fibers wound helically to mimic the architecture of the collagen fibers in natural tissues.[27] The main goal was to provide the possibility of tailoring the rheological response of these synthetic systems to reproduce and sustain the mechanical characteristics of natural ligaments. The motivation is associated with the fact that the viscoelastic characteristics of the ligament regulate important biomedical functions of the knee during dynamic loading.[28–30] Dynamic tests have also been widely used to study the effect of composition on the glass transition temperature and mechanical behavior of bone cements. A new concept includes the use of biodegradable and hydrophilic materials in the formulations[31,32] that can induce bone formation inside the cement during degradation; their fluid uptake capability enhances the compatibility with the biological environment and improves the fixation of the prosthesis (by press-fitting). Finally, examples of biomaterials for hard tissue replacement could include polyethylene systems, especially if combined with bioactive fillers such as hydroxyapatite. These composites have been considered as bone analogue materials.[33] Dynamic mechanical tests were performed in order to look at the effect of the particle size on the viscoelastic features of the composite.[34] The solid-state rheological properties in polyethylene systems are mostly dependent on the α_c relaxation, characterized by both dynamic and creep tests on injection-molded parts.[35] This relaxation is intimately associated with the existence of the crystalline phase and may be seen in other semicrystalline polymers, such as polyvinylidene fluoride; in this case, the α_c relaxation will also determine, for example, the creep behavior of this material (discussed in Section 9.4).[12] In addition, hydroxyapatite may be used to reinforce biodegradable systems, such as polylactides, enabling an increase in osteoconductivity. Such composites were also characterized by dynamic tests.[36] As expected, E' increases as the ceramic content increases, because the ceramic is stiffer than the thermoplastic. At the same time, tan δ decreases because the filler is essentially elastic (the tan δ of hydroxyapatite is much lower than the polymer). Therefore, as the damping of the resulting composite may be given approximately by a simple mixture rule, the resulting composite exhibits lower tan δ than the pure polymer.

9.7 MECHANICAL TESTS IN SIMULATED PHYSIOLOGICAL SOLUTIONS

For obvious reasons, there has been an increasing interest in performing mechanical tests while the sample is immersed in solutions that simulate their environment in physiological conditions. Therefore, such solutions should mimic the characteristics of body fluids in terms of temperature, pH, ionic strength, and composition. In some cases, the solution could include proteins and enzymes. Proteins are relevant because they will be quickly adsorbed onto the material surface and (especially in systems with high surface area such as membranes or foams) it may affect their mechanical properties and change the degradation profile. For biodegradable materials, the inclusion of enzymes may accelerate the degradation process, and this may be monitored by mechanical tests with the samples immersed in solutions. Dynamic tests have an important role here, as the stress levels are sufficiently low to follow the change with time of the mechanical properties of a sample without destroying it. A further contribution in such an experiment would be the continuous monitoring of the mechanical properties of biomaterials in the presence of cells, which would have particular relevance in the tissue engineering field. Bioactivity tests can also be complemented by following the mineralization of the specimen in simulated body fluid (SBF)[37] that will continuously affect the mechanical/viscoelastic properties of the substrate. For example, the material is immersed during different periods of time in SBF and then tested using dynamic, creep, or conventional quasi-static analysis. It is expected that mineralization leads to a stiffer material, and due to the essentially elastic nature of the mineral phase, it should exhibit lower damping capability.

Two possible methods exist for following the mechanical properties of materials with time:

1. On-line measurements imply that the sample is always immersed while the mechanical tests are done. In this case, for example, dynamic tests allow one to monitor the degradation process in the same sample, which should exhibit, in most cases, a continuous decrease of stiffness. Exceptions may appear in materials that contain releasable plasticizer molecules; in such a case, the dissolution of such specimens may increase the modulus of the material.[38]
2. Off-line measurements imply that the samples are immersed in an external bath and, from time to time, are tested in the machine. This saves equipment time and should be done in long-term tests.

Some examples will be given here that explore both tests.

Starch-based materials, particularly if blended with other synthetic polymers, have been shown to have great potential in a series of biomedical applications, such as scaffolds for the tissue engineering of bone and cartilage, membranes, materials for bone fixation and replacement, carriers for the controlled release of drugs and other bioactive agents, and new hydrogels and partially degradable bone cements. A list of references with such applications may be found in Reference 24. Other chapters of this book also discuss the application of such starch-based blends in regenerative medicine.

In Figure 9.8, an example is presented showing the evolution of E' and E'' with time of initially dry samples of starch-based blends, processed with different conditions, after being immersed in an isotonic saline solution at 37ºC.[39] These materials are hydroscopic, and the swelling kinetics may be followed by looking at the decrease of E'. As predicted, the material processed with shear-

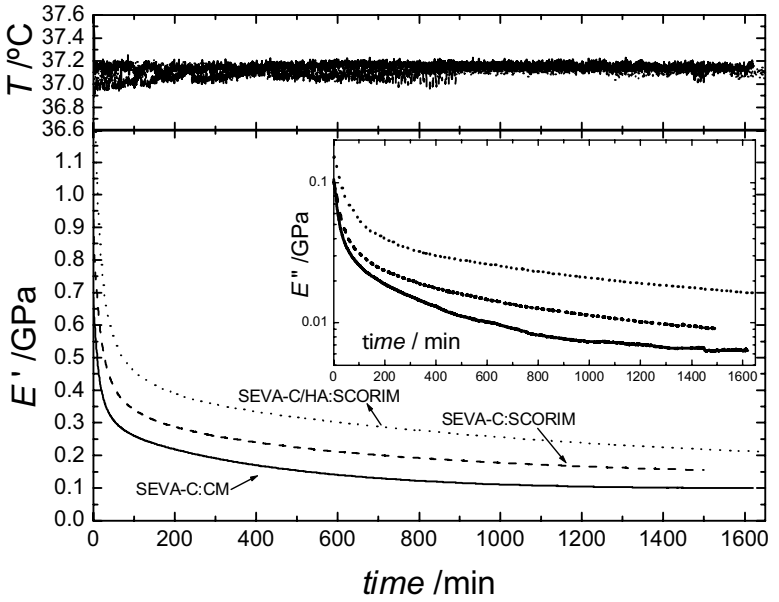

FIGURE 9.8 Dynamic mechanical analysis on injected–molded pieces of blends of starch and a copolymer of ethylene and vinyl alcohol (SEVA-C), tested at 37ºC in an isotonic saline solution, performed in a Perkin-Elmer DMA7 apparatus. The samples are initially in the dry state and the tests were performed at 1 Hz, using the three-point bending mode. SEVA-C:CM — material processed with conventional injection molding; SEVA-C:SCORIM — material processed with shear-controlled orientation in injection molding; SEVA-C/HA:SCORIM — SEVA-C previously compounded with 30% of hydroxyapatite. The top graphic presents the temperature reading as a function of time. (For more details, see Mano, J.F. and Reis, R.L., *Mat. Sci. Eng. A*, 370, 321, 2004.)

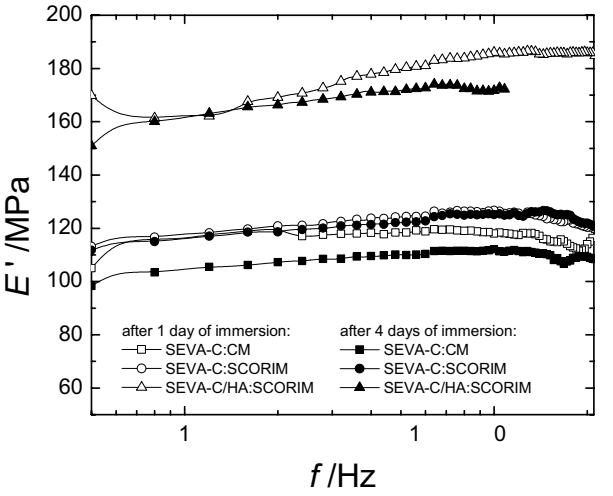

FIGURE 9.9 Frequency scan tests on the materials of Figure 9.8 after 1 (open symbols) and 4 (closed symbols) days of immersion in an isotonic saline solution. (For more details, see Mano, J.F. and Reis, R.L., *Mat. Sci. Eng. A*, 370, 321, 2004.)

controlled orientation in injection molding is stiffer than the one processed by conventional injection molding. The storage modulus increases further in the blend that is reinforced with hydroxyapatite. It was found that the composite shows higher tan δ in the entire timescale, which was attributed to energy dissipation through frictional effects in the interface between the thermoplastic and the ceramic.[39] Figure 9.8 also shows the variation of the measured temperature during the experiments, which was found to be quite stable.

Off-line experiments are exemplified in Figure 9.9, performed on the same materials as in Figure 9.8. Here, frequency scans are done while the samples are immersed in an isotonic saline solution at 37ºC, after 1 day and 4 days of immersion, in an external bath.[39] The decrease of stiffness in the studied frequency range was attributed to the swelling that may occur after 24 hours. As expected, the composite maintained higher values of E' after these two immersion time steps. Such results clearly point out the necessity of performing mechanical tests in hydrophilic biomaterials in simulated physiological conditions, as they exhibit clear differences in such conditions compared with their dry state.

Besides dynamic tests on such starch-based materials, creep tests in immersion were done in formulations that were found to be suitable for use as bone cements.[32] Such experiments are important to predict the behavior of such hydrophilic materials in clinical conditions and to determine which developed formulations are more susceptible to creep. In that work,[32] recovery studies were also performed to find the irrecoverable component of the strain resulting from pure viscous effects. The developed materials were also subjected to dynamic tests in wet conditions, both by frequency and temperature scans. These sets of experiments provided a broad view of the viscoelastic features of such systems, which were compared with a commercial formulation. Such kinds of dynamic experiments were also performed in more conventional bone cement systems.[21]

9.8 CONCLUSIONS

Different mechanical characterization tests that are particularly suitable for evaluating the behavior of materials for biomedical applications were reviewed. Conventional quasi-static or fatigue tests are very popular, providing the stiffness and strength of the material, as well as its long-term mechanical resistance under cyclic loads. As tissues exhibit a viscoelastic character, materials in

contact with them should have the same time-dependent mechanical behavior (viscoelastic biocompatibility). Therefore, this chapter focused on the use of some techniques (e.g., creep and dynamic tests) that allow one to monitor the solid-state rheological properties of materials. Special attention was given to measurements that probe the properties of materials or devices while immersed in simulated physiological conditions. This allows one to have a better perception of the materials' behavior under realistic conditions.

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10 Chitosan-Based Microcomposites — From Biodegradable Microparticles to Self-Curing Hydrogels

Alberto Gallardo, María Rosa Aguilar, Carlos Elvira, Carlos Peniche, and Julio San Román

CONTENTS

10.1 INTRODUCTION

Chitosan (CHI), a basic (ionizable), linear polysaccharide composed of *N*-acetyl-*D*-glucosamine and *D*-glucosamine units linked by β-*D* (1→ 4) bonds (see Figure 10.1), is obtained by partial *N*-deacetylation of chitin (the *N*-deacetylation is almost never complete). Chitin is the second most abundant natural polysaccharide on the earth next to cellulose. It is present in the exoskeleton of crustaceans, cuticle of insects, and wall of fungi. CHI itself is also present in some microorganisms and fungi.

The term *chitosan* refers in fact to a series of deacetylated chitins with different molecular weight (50 kDa to 2000 kDa), viscosity, and degree of *N*-deacetylation (40 to 98%). Scant attention has been paid to the parent homopolymer, chitin (in spite of its structural similarity to cellulose), primarily due to its inertness. Chitin is a highly insoluble material and possesses a low chemical reactivity. CHI, on the other hand, offers many possibilities to a material chemist or bioengineer. One of the most astonishing facts about CHI presented by the different scientific publications is the huge versatility of structures and applications of the CHI-derived materials. CHI and CHI derivatives can be easily fabricated into fibers, films, porous scaffolds, hydrogels, and micro- and nanospheres, which are highly interesting for manufacturing many different medical devices.[1,2] In addition, CHI structure makes it possible to easily incorporate it in composites or supramolecular

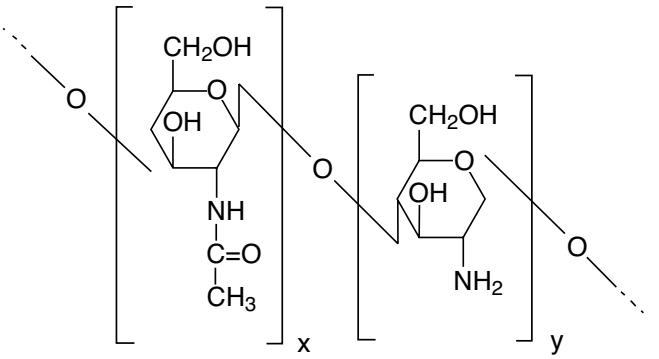
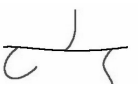
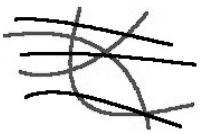


FIGURE 10.1 Schematic structure of chitosan (CHI).

conjugates. Interpenetrating networks, polyelectrolyte complexes, graft copolymers, blends, or other structures can be prepared, as shown in Table 10.1.

This highly basic polysaccharide presents a number of ionizable primary amino groups readily available for chemical reaction and salt formation with acids. CHI, therefore, undergoes reactions typical of amines, of which *N*-acetylation and Schift reaction are the most important. Glutaraldehyde has been extensively used as a CHI crosslinking agent because it retards the biodegradation of the polysaccharide and enhances its structural stability. This aldehyde, as well as other coupling agents, also allows the preparation of interpenetrating polymer networks, IPNs (two chemically independent

TABLE 10.1
Possible CHI-Based Structures and Associates

Type of Association	Scheme	Structures
Ionic bonds		Polyelectrolyte complex (PEC)
Covalent bonds		Interpenetrating polymer network (IPN)
		Semi-IPN
		Grafting
Mixing (other interactions can take place)		Blends
		Hybrid matrices

networks that are physically entangled), and semi-IPNs (semi-interpenetrating polymer networks composed of linear uncrosslinked macromolecules imbibed in an infinite CHI network), as well as the simultaneous crosslinking of CHI and proteins. CHI, like other polysaccharides, also has the availability of reactive hydroxyl groups in its structure, which can also be derivatized. CHI derivatives are easily obtained under mild conditions and can be considered as substituted glucans, and they offer a wide range of design possibilities as composite or conjugate components.

CHI is insoluble at alkaline and neutral pH. As pH decreases, amine groups of CHI become protonated, with a resultant positively charged soluble polysaccharide ($R\text{-}NH_3^+$) and an extended configuration because of the ionic repulsion between cationic groups. As a consequence of this behavior, many CHI-based matrices are pH sensitive and become very attractive for the actuation (for instance) at the acid pH of the stomach. Derivatizations, or interactions that affect the population of NH_2 groups, will influence, of course, this pH dependency. A CHI-based material without free amino groups will not exhibit any pH sensitivity unless the other components do. The aqueous solubility of CHI, anyway, depends on the degree of *N*-deacetylation. CHI with a low degree of *N*-deacetylation (40%) has been found to be soluble up to pH 9.0, whereas CHI with a degree of *N*-deacetylation of about 85% is soluble only up to pH 6.5. CHI salts (glutamate, chloride, etc.) are water-soluble.

Cationicity of CHI (at acid pH) allows the formation (in aqueous environment) of polyelectrolyte complexes (PEC) by electrostatic interactions with water-soluble anionic macromolecules like DNA, glucosaminoglycans (GAGs), proteins, or synthetic anionic polymers. The formation of these complexes strongly affects the polymer physical properties such as solubility, rheology, conductivity, and turbidity of the polymeric solution. The complex stability is dependent on variables such as charge density, solvent, ionic strength, pH, and temperature.[3] These PECs, which are obviously formed at acid pH, usually are not water-soluble (because one of the components may become neutral) and, if the ionic interactions are strong enough, they may be stable at physiological pH (where the free amino groups become neutral).[4] In any case, the pH of the medium can modulate these ionic interactions and, subsequently, the PEC properties.

This family of polymers, CHI, is extensively employed in the industry as a flocculant in the clarification of wastewater, as a chelating agent for many transition metal ions for the detoxification of hazardous waste, for the clarification of beverages, for agricultural uses, and in the paper and textile industries. In the cosmetic industry, CHI has been used in the fabrication of hair care, skin care, and oral care products.

As biomaterial, CHI has attracted a great interest in medicine and pharmacology because of its biodegradability, biocompatibility, and the wide range of reported positive biological responses and activities. It has been used or proposed in the fabrication of drug delivery devices,[5] in cell encapsulation,[6] in orthopedics, in wound healing,[7] in adhesive formulation for surgical applications,[8,9] in ophthalmology (such as contact lens coatings, contact lens materials, or artificial tear fluid),[10] in pharmaceuticals,[11] in dentistry[12] and bone healing,[13] as hemostatic agent, as wound healing accelerator,[14] in the fabrication of scaffolds for tissue engineering (making use of its cell affinity; it has exhibited osteo- and neochondriogenic properties), as hypocholesteremic agent, as nonviral DNA vector, or in mucosal delivery systems (it is a mucoadhesive and mucosal penetration enhancer). It is also fungi- and bacteriostatic.

This chapter is devoted to illustrating the enormous possibilities of incorporating CHI in complex systems (Figure 10.1) as promising structures for biomedical applications. We have classified the CHI derivatives as a function of the nature of the partner component, protein, polysaccharide, DNA, degradable polyester, inorganic, synthetic (ionic or nonionic) polymer, or lipid.

10.2 CHITOSAN/PROTEINS

Chitosan in its cationic form can ionically interact with most of the proteins, which exhibit an amphoteric character. Some polyelectrolyte complexes (PEC) between chitosan and proteins such

as gelatin have been described.[15] If there is no ionic interactions, polar interactions like hydrogen bonding can still take place between these polar macromolecules of natural origin. The point is that compatible and resorbable chitosan/protein associates (obtained without covalent conjugation) can be easily obtained by mixing solutions (at acid pH) followed by casting or freeze-drying. In addition, chitosan and proteins can be simultaneously crosslinked with glutaraldehyde[16] or analogues to obtain covalently conjugated matrices, which are still biodegradable. Other specific covalent links such as the ones catalyzed by the enzymes transglutaminase and tyrosinase have been described.[17]

Although some chitosan/albumin,[18–20] chitosan/fibroin (blended with PVA for wound healing),[21] or chitosan/keratin (that seems to be also a good substrate for mammalian cell culture, because of supported fibroblast attachment and proliferation)[22] matrices can be found in the literature, collagen and gelatin (the partially denaturized form of collagen) are, without doubt, the most interesting and reported proteins to be conjugated or blended with chitosan, mainly for tissue engineering applications. Collagen is the major component of the extracellular matrices (ECM) of human tissues, and chitosan is considered to be a GAG analogue (other main ECM component); therefore, collagen/chitosan or gelatin/chitosan matrices have been proposed and studied as biodegradable scaffolds in tissue engineering.[23,24] The composition and the preparation method can control (to some extent) the swelling, the mechanical properties, and the biodegradation rate, which can be tailored to mimic the properties of native tissue.

Especially relevant are the efforts directed toward the preparation of artificial skins similar to the commercial Apligraft®, which is a collagen-based dermal and epidermal combined substitute that incorporates allogenic fibroblasts and keratinocytes. A collagen/GAG/chitosan-based human skin equivalent was already developed in the early 1990s,[25–27] and a gelatin/chitosan artificial skin (crosslinked with glutaraldehyde) has been recently proposed.[28] Fibroblasts and keratinocytes have been cocultured in porous asymmetric scaffolds, mimicking the structure of the natural skin. They are promising skin substitutes, and some clinical uses have been reported.

Gelatin/chitosan or collagen/chitosan supports (some of them crosslinked with glutaraldehyde) have also been investigated (with promising results in cell growth and proliferation) in tracheal,[29] cartilage,[30] nerve,[20,31,32] or bone tissue[33] repair and regeneration. In this last example, a composite with hydroxyapatite was used. Other modification can be the incorporation of hyaluronic acid (a GAG) in crosslinked chitosan/gelatin membranes, improving fibroblast adhesion.[34] Some studies on hepatocyte attachment for bioartificial liver organoids have also been reported.[19]

In drug delivery, chitosan itself has been proposed as carrier for protein delivery (vaccines, etc.), mainly for mucosal administration. In addition to this, chitosan/gelatin conjugates have been investigated as carriers of different drugs.[35] Chitosan/gelatin microspheres (ionically crosslinked with tripolyphosphate) have been proposed for stomach-specific drug delivery.[36]

10.3 CHITOSAN/POLYSACCHARIDES

Ionized chitosan (at pH below 6.5) can ionically interact (in aqueous media) with hydrosoluble anionic polysaccharides to form biodegradable polyelectrolyte complexes (PECs) of natural origin, which have attracted great interest in recent years (in the biomedical and pharmaceutical fields). The recent literature has reported the design, for biomedical purposes, of different polyelectrolyte complexes (PEC) formed between chitosan and different anionic polysaccharides such as dextransulfate,[37–39] pectin,[40–42] carboxymethylcellulose,[43–45] xanthan,[46,47] and mainly alginate and GAGs (chondroitin sulfate, hyaluronic acid, or heparin), which will attract special attention in this section.

Alginate contains glucuronic acid residues that show affinity for polyvalent cations (like calcium), leading to coacervation or to gel formation because units of different macromolecules can participate simultaneously in the interaction. This process allows the entrapment of active compounds or even cells by dipping the mixture with alginate in calcium (or other polyvalent cation)

solutions. The droplets instantaneously solidify, forming a gel and entrapping inside the active entity. Alginate/calcium systems are widely used in pharmaceutical preparations, cell encapsulation (bacteria, yeasts, etc.), and enzyme immobilization. In biomedical applications, encapsulation interest switched in recent years to the preparation of bioartificial organs by mammalian cell encapsulation and immunoisolation, like the encapsulation of islet of Langerhans for the development of bioartificial pancreas (for the treatment of diabetes mellitus). However, in this application, alginate beads fail in some specific requirements (mainly mechanical resistance). Different alginate derivatives have been reported trying to improve its performance. Since the initial description by Lim and Sun in 1980,[48] many efforts have been dedicated to the development and evaluation of alginate/Ca^{2+}/polylysine capsules (a multicomponent PEC). Other alginate derivatives have been proposed with this purpose (mammalian cell encapsulation), chitosan/alginate (or chitosan/alginate/calcium) being one of the first combinations chosen. Besides islets,[49–51] hepatocytes (with good survival and immunoisolation behavior),[52,53] yeast cells (saccharomyces cerevisiae),[54] seeds (showing a high viability),[55] enzymes,[56] antibodies,[57] insulin,[58] and other proteins (for oral administration),[59–61] adriamicin (for arterial embolization of microcapsules),[62] and different drugs (also for oral administration intending to overcome the release in the stomach)[63–65] have been immobilized or encapsulated in chitosan/alginate derivatives.

Chitosan/alginate PECs have been used not only for encapsulation purposes. Other morphologies or structures have been described. Porous alginate/Ca^{2+}/galactosylated chitosan three-dimensional sponges providing specific hepatocyte recognition for cell attachment (to manufacture artificial liver) have been recently reported.[66] Other sponges have been proposed as suitable matrices for tissue engineering or wound dressings.[67,68] Lim et al. have studied the film formation by casting from chitosan/alginate (or chitosan/alginate/Ca^{2+}) suspensions or coacervates for wound dressings and membrane coating.[69–72]

Glycosaminoglycans (GAGs) are anionic polysaccharides (in nature they are attached to core proteins forming the proteoglycans, which have a key role in the organization and functioning of the extracellular matrix), which are natural components of human tissues. These polysaccharides — mainly hyaluronic acid, chondroitin sulfate, and heparin — have attracted a great interest as biocompatible and biodegradable components of pharmaceutical and medical devices. These compounds bear glucosamine or *N*-acetylglucosamine residues, the two components of CHI. Therefore, chitosan being a GAG analogue itself with demonstrated good cytocompatibility, chitosan/GAG compounds have been evaluated as supports in tissue engineering, mainly chitosan/chondroitin sulfate. Chitosan/chondroitin sulfate membranes have been shown to support chondrogenesis,[73] being promising materials for cartilage repair. Both polymers, together with carboxymethylcellulose and polygalacturonic acid, have been used to encapsulate rabbit hepatocytes in preliminary studies for extracorporeal liver support. The hepatocytes maintained viability and functionality after 6 days.[74] Platelet-derived growth factor releasing chitosan/chondroitin sulfate sponges have been also evaluated in bone regeneration.[75] As it was described in the previous section, a human skin equivalent composed of collagen/GAG/chitosan was reported by Damour et al. more than 10 years ago.[26,27] This group was one of the first in investigating the ionic interactions between chitosan and the anionic GAGs hyaluronic acid and chondroitin sulfate.[76] They found in any case a better cell attachment and proliferation with pure chitosan.[77] In fact, chitosan/GAG materials (including chitosan/heparin, another GAG with very relevant biological properties) have been evaluated as modulators of the proliferation of vascular cells.[38]

Hyaluronic acid presents a higher mucoadhesivity than chitosan, which is considered a mucoadhesive and mucosal penetration enhancer. Combination of both has shown to be highly mucoadhesive[78,79] and to synergistically enhance nasal drug absorption.[80] It has been proposed as an advantageous formulation for mucosal delivery. Chitosan/chondroitin sulfate complexes have also been reported as biocompatible and biodegradable vehicles for drug delivery. Chondroitin sulfate modulates both the degradation and the release rate of chitosan or chitosan/alginate beads.[81]

Heparin/chitosan PECs have been described as attractive matrices for wound healing, due to the interaction and stabilization of heparin with growth factors involved in that healing process.[82–84] Heparin has been also incorporated or immobilized in chitosan/PEG derivatives conferring antithrombogenicity,[85] in chitosan/EVA matrices intending to prevent calcification or thrombosis,[86,87] in chitosan/polylactic acid microspheres to prevent restenosis,[88] or in chitosan/PEG–alginate microspheres for the prevention of thrombosis.[89]

Other combinations of chitosan and polysaccharides, including the nonionic ones, are found in the drug delivery field, such as the chitosan-coated ethylcellulose microparticles for prolonged intestinal absorption,[90] the development of chitosan–ethylcellulose or chitosan–cellulose acetate butyrate multicore microparticles (chitosan being the inner core) for oral delivery,[91] the preparation of chitosan–ethylcellulose mucoadhesive bilayered devices for buccal drug delivery,[92] or the encapsulation of dextran–doxorubicin conjugate in chitosan nanoparticles for the treatment of solid tumors.[93]

10.4 CHITOSAN/DNA

Anionic DNA can ionically interact with cationic chitosan, giving rise to complexes that are very interesting as nonviral gene delivery agents. In efficient gene delivery, plasmid DNA has to be introduced into target cells and transcribed, and the genetic information ultimately has to be translated into the corresponding protein. Viral gene delivery yields high transfection efficiency, but presents some serious drawbacks such as inflammatory and oncogenic effects. For this reason, research on this topic also focus on nonviral carriers,[94] which are mainly cationic polymers or cationic phospholipids able to electrostatically interact with the anionic DNA to form complexes (polyelectrolyte complexes [PEC] in the case of cationic polymers). Entrapped DNA in the PECs is shielded from contact with DNAses.[95] Among cationic polymers, chitosan is very attractive because of its natural origin, biocompatibility, low toxicity, degradability, and high affinity for cell membranes and because it is a good vehicle for mucosal delivery. In addition, the cationic nature seems to be related to the cell membrane adhesion (in contrast with naked anionic DNA) and lysosomal escape, and the complex appears to be stable until it has entered the cell. Consequently, chitosan is a very attractive vector for gene delivery. Excellent reviews can be found in the recent literature.[96–98] Since the first chitosan/DNA complex for gene delivery was proposed in 1995,[99] the main efforts have been directed toward overcoming the low transfection efficiency. Today, it is well accepted that size and shape are key parameters. In fact, the nanosized chitosan/DNA spheres developed by Leong et al. were the first ones (to our knowledge) showing positive transfection,[99,100] in contrast with the nonefficient complexes described previously of higher size or nonspherical shape. Other similar nanoparticles have been described since then,[101–105] although current data related to the influence of the different factors are still contradictory. In addition, the influence of the molecular weight of chitosan and other parameters in the transfection efficiency has been demonstrated.[106,107]

Besides, it is possible to incorporate to the vectors other designs or active compounds. Chloroquine (a lysosomolytic agent) has been added to some formulations, but the results are quite doubtful. Target-specific ligands such as galactose or transferrin have been covalently linked to the polysaccharide chain. Galactosylated chitosan-graft-dextran/DNA,[108] galactosylated chitosan-graft-PVP/DNA,[109] and galactosylated chitosan-graft-PEG/DNA[110] complexes have shown a good specificity to liver cells, which have the specific receptors for galactose. The transferrin receptor responsible for iron import to the cells is found on many mammalian cells. Transferrin ligand has been used to transfer different drugs and DNA, and recently it has been coupled to chitosan/DNA nanoparticles.[104] Quaternized oligomers of trimethylated chitosan[111] and deoxycholic acid modified chitosan[112] have also been described as nonviral DNA vectors. On the other side, since chitosan has been shown to be a key component in mucosal delivery systems, oral vaccine designs (based on DNA) have been reported, mainly intended for nasal administration.[113–115]

10.5 CHITOSAN/DEGRADABLE POLYESTERS

Poly(hydroxyacids) such as polylactic acid (PLA), polyglycolic acid (PGA), poly-ε-caprolactone (PCL), or the corresponding copolymers (PLGA, etc.) have been widely investigated in the drug delivery and tissue engineering fields. These materials present very good properties, such as biodegradation, nontoxicity of the residual products, feasibility of making 3-D porous scaffolds, and good mechanical properties, among others. The biodegradation rate can be modulated by the proper choice of the macromolecular components.

These materials, however, fail in some specific needs. In tissue engineering, their surfaces are hydrophobic and do not promote cell adhesion and proliferation. A convenient method to improve the cytocompatibility of these polyhydroxyacids-based scaffolds or matrices (maintaining their good block properties) is to introduce a cytocompatible layer of chitosan (also biodegradable) on the polymer surface. This layer can be introduced by simple coating or by covalent immobilization. Different coupling methods to covalently attach chitosan on PLA surfaces can be found in the literature.[116–120] In these works, an increased cytocompatibility with osteoblast or chondrocytes was reported. On the other side, chitosan or chitosan derivative coatings can be obtained by different methods. Chitosan–amino acid conjugates, which can be considered as GAG analogues, can coat PLA membranes by simple dipping, promoting chondrogenesis.[121] Coating can be obtained also by interpolyelectrolyte complexation (layer-by-layer assembly) through the introduction of free amino groups on the surface of PLA membranes and using polystyrene sulfonate as an anionic intermediate layer. The deposited chitosan improved cytocompatibility to human endothelial cells.[122]

Surface modification is not the only described method to obtain useful polyester/chitosan conjugates with improved cytocompatibility for tissue engineering. Porous PGA/chitosan matrix can be fabricated by mixing solutions in DMSO and acidic water, respectively, followed by freezing, immersion in NaOH, washing with phosphate buffer, and drying.[123] Porous chitosan/PLA or chitosan/PCL microcomposite matrices have been prepared by emulsifying aqueous chitosan in organic PLA or PCL solution followed by freeze-drying.[13,124] These microcomposites were loaded with a growth factor (GF) and proposed as a superior strategy in bone regenerative therapy, associated with the combinative use of chitosan-based scaffolds (osteoconductive) and the controlled release of GF.

In the drug delivery field, chitosan has been selected as coating material of polyester devices for different purposes. Chitosan coating may lead to a control or modulation on the drug release from PLA or PCL microparticles.[125,126] Injectable chitosan-coated PLA or PLA/PGA blend microspheres have been proposed for the controlled release of cisplatin (an antiproliferative) or 5-fluorouracil in the treatment of restenosis after angioplasty or vascular injury[88,127] and cerebral tumors, respectively.[128]

In oral drug administration, chitosan is very attractive because of its recognized mucoadhesivity and ability to enhance the penetration of large molecules across mucosal surfaces.[129] It has been described that chitosan-coated PLGA nanoparticles enhanced the nasal transport of the encapsulated protein tetanus toxoid (a vaccine)[130] compared with control formulations. Similar nanospheres improved mucoadhesion and drug absorption of peptide delivery system.[131] In oral delivery, composite membranes or multilayer films obtained by emulsification/casting and casting processes, respectively, have been proposed as dosage forms for the periodontal release of ipriflavone.[132]

10.6 CHITOSAN/INORGANICS

Chitosan/inorganic composites are very attractive hybrid materials, since nature itself successfully uses inorganic–organic composites, especially for structural purposes.[133] Probably the most described composites with chitosan are those based on calcium phosphates, e.g., hydroxyapatite (HA) or β-tricalcium phosphate (β-TCP). These bioceramics are excellent candidates for bone repair and regeneration since their chemical compositions are similar to the inorganic components

of bone. In this sense, calcium phosphate cements are well-known orthopedic materials for bone filling. After implantation, they are rapidly integrated into the bone structure and finally transformed into new bone. These cements can be molded during the operation and are injectable.

Chitosan itself has been also described as a promoter of bone formation.[134] Composite materials can incorporate favorable properties from both components. Calcium phosphates provide matrix reinforcement and osteoconductivity, while chitosan incorporates biodegradability and flexibility between others. Different templates, porous scaffolds, films, or bone cements have been described with these purposes. Composites can be obtained by mixing, coating,[135] or precipitation (from calcium and phosphate solutions).[136] Usually, the insoluble ceramic powder (finally divided) is just suspended in the preparation media that contains chitosan and then treated. In the case of porous scaffolds (sponge-like), which are very interesting matrices for bone tissue engineering, they can be obtained by simple freeze-drying.

The incorporation of chitosan to bone cements intends to improve or modulate properties such as injectability, degradation rate, or mechanical performance. An injectable chitosan–calcium phosphate bone filler that set in physiological conditions but not *in vitro* has been recently described.[137] They used an initial suspension at pH lower than 6.5 where the amino groups are ionized and chitosan is water-soluble. After injection or implantation in the body, the paste-like moldable/injectable system undergoes gelation because of the pH increase (to physiological 7.4), and the amino groups become neutral. In this process, the ceramic is physically entrapped inside the gel matrix. The preliminary results indicated that this hybrid material can be a good candidate for injectable, resorbable (in the case of TCP) scaffolds for bone tissue regeneration in non-load-bearing applications. In other work, chitosan used as an adjuvant of injectable calcium phosphate cements was shown to improve injectability, to increase setting time, and to limit the evolution of the cement toward HA by maintaining the octacalcium phosphate phase.[138] Yokoyama et al.[139] investigated a composite cement where chitosan was administered in a mixture with citric acid and glucose, providing setting times from 5.5 to 6.4 min. When incubated in physiological saline, the cements were transformed into HA with compression strength of 15 to 20 MPa. After 4 weeks, the inflammation disappeared and the cement was bound to bone. Some chitosan covalent derivatives have been studied also as cement modifiers. Phosphorylated chitosan has been used to reinforce calcium phosphate cements,[140] which have been tested in rabbit bone repair with good results. Calcium phosphate cements have been also modified with chitosan lactate to lower rigidity for periodontal purposes.[12]

Besides cements, some efforts have been put into the preparation of porous three-dimensional scaffolds of chitosan–calcium phosphate composites for bone tissue engineering. Zhang et al. have recently published different papers on that.[141–143] They prepared the sponges by freezing at –20ºC, which allows a solid–liquid phase separation and lyophilization. Calcium phosphate greatly reinforced the chitosan matrix and also modulated the burst effect on the gentamicin release when loaded. A good cellular biocompatibility was also observed. This group has also reported the preparation of macroporous bioceramics by nesting chitosan sponges for load-bearing bone implants.[144] Chung at al. have reported the preparation of alternative chitosan–tricalcium phosphate sponges, in some cases loaded with platelet-derived growth factor, also by mixing and freeze-drying.[145,146] Osteoblast growth and differentiation, as well as the osteogenic effect of the growth factor release in the loaded scaffolds, were observed. Other porous composite matrices can be obtained by a very different approach: After an initial chitosan crosslinking (with glutaraldehyde), the obtained hydrogel is loaded with the calcium phosphate by alternative soaking in $CaCl_2$ and Na_2HPO_4 aqueous solutions (leading to the precipitation of the calcium phosphate *in situ*), freeze-dried, and finally subjected to enzymatic hydrolysis to achieve the pore formation.[147]

Porous calcium phosphate coating over phosphorylated chitosan films[135] (obtained biomimetically by hydroxyapatite nucleation) and composite chitosan/hydroxyapatite membranes have also been reported.[148] In the latter, biocompatibility studies (by implanting in rats) revealed a biologically good toleration, with fibrous encapsulation and occasional osteogenesis.

Chitosan containing glycerol-2-phosphate undergoes sol-gel transition at a temperature close to 37ºC, which makes this material very attractive as injectable biomaterial or drug vehicle.[149] A commercial injectable formulation has been developed by BioSyntech. Other formulation of interest in drug delivery is the one obtained by ionic complexation of chitosan and tripolyphosphate. Microparticles[150,151] and beads for colon delivery of macromolecules (where chitosan is enzymatically degraded)[152] have been investigated.

Siliceous composites have also been described. Semi-IPN membranes of chitosan incorporated in tetraethylorthosilicate silicon alkoxyde (TEOS) network exhibited pH dependence in swelling and drug release.[153] Similar hybrids have been prepared in the form of transparent films.[154] Chitosan/poly(aminopropylsiloxane) blends have also been reported.[155]

10.7 CHITOSAN/SYNTHETIC HYDROPHILIC POLYMERS (NONIONIC)

Nonionic water-soluble synthetic polymers such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), or polyvinylalcohol (PVA) have many biomedical applications. They are biocompatible and nontoxic and are common components of hydrogels.

The combination of chitosan with these hydrophilic polymers can be simple blends (made by mixing aqueous solutions at acid pH, water removal, and maybe further neutralization), graft copolymers (of the hydrophilic chains on the NH_2 group of chitosan), or semi-IPNs (either noncrosslinked hydrophilic chains in a chitosan matrix crosslinked with glutaraldehyde or other coupling agent, or (we can call these inverse semi-IPNs) chitosan uncrosslinked chains embedded in a crosslinked hydrophilic network usually obtained by *in situ* polymerization using appropriate difunctional monomers. True IPNs can also be obtained by simultaneous crosslinking of both structures.[156] These combined structures frequently behave as hydrogels, and swelling may or may not keep the pH sensitivity of the original chitosan chain (depending on the presence of these NH_2 functionalities after the preparation method), associated with the ionizable NH_2 group. If pH sensitive, the hydrogel swells more in acidic environment and for this reason can be appropriate to deliver drugs into the stomach. These structures may intend to improve or modulate certain properties of one of the individual components (from the point of view of chitosan, hydrophilicity, and some mechanical properties) or may intend to obtain a synergistic effect of both components.

Risbud et al. have prepared and evaluated compatible hydrogel membranes made of chitosan/PVP semi-IPNs (chitosan crosslinked with glutaraldehyde). The membranes, which swell 60% and are cytocompatible,[157] immunocompatible, and pH sensitive, have been studied as potential candidates in islet immunoisolation,[158,159] transplantation,[160] wound healing,[161] and antibiotic release.[162] This group has also reported the preparation of (inverse) chitosan/polyacrylamide semi-IPN by the polymerization of acrylamide in the presence of chitosan and using *N,N'*-methylene bisacrylamide as acrylic difunctional crosslinker.[163] The resulting hydrogels, which were shown to be biocompatible, swelled considerably, and did not exhibit any pH dependence, were evaluated *in vitro* for antibiotic delivery.

There are also examples of the simultaneous use of PEG and PVP with chitosan. pH-sensitive semi-IPN hydrogels prepared by polymerization of vinyl pyrrolidone, *N,N'*-methylene bisacrylamide, and an acrylic derivative of PEG in the presence of chitosan have been reported recently.[164] PVP and PEG grafted on galactosylated chitosan (not simultaneously) have been proposed as hepatocyte-targeting DNA carriers, as described in the DNA section.[109,110] PEG and PVP were used in this work as hydrophilic modifiers. In this sense, PEG is a well-known molecule used as surface modifier of micro- or nanoparticles or to derivatize proteins (this is called pegylation) to make them stealth particles that avoid a rapid recognition and uptake by the immune system. Several pegylated proteins are already on the market.

The preparation of different chitosan/PEG semi-IPNs has been described in recent years. In addition to glutaraldehyde, other coupling agents have been successfully used, such as glyoxal[165]

or sugar-based agents.[166] These semi-IPNs are swellable hydrogels that exhibit a good cytocompatibility and pH sensitivity (one of the hydrogels has been proposed for the stomach delivery of antibiotics).[167] The matrix crosslinked with the sugar derivatives has been loaded with calcium phosphate, fabricated as porous three-dimensional structure by freeze-drying methods, and proposed as scaffold for healing cartilage.[168] As mentioned in previous sections, Chandy et al. have developed and evaluated antithrombogenic heparin immobilized chitosan–PEG microbeads[85] (semi-IPN where chitosan was crosslinked with glutaraldehyde), which could be useful as matrices in hemoperfusion,[169] and alginate chitosan (also crosslinked with GA)/PEG microcapsules as semipermeable membranes in cell encapsulation.[6] Methoxy-PEG end capped with a sulfonic moiety has been used to prepare polyelectrolyte complexes with chitosan for blood-contacting applications.[170]

Simple blending of chitosan and PEG leads to materials with some improved mechanical properties, such as ductility,[171] cytocompatibility,[172] or the permeability and blood compatibility for hemodialysis purposes.[173] Graft copolymers (PEG chains grafted on chitosan chains) exhibiting also an improvement in mechanical properties[171] or water solubility have been also synthesized.[174]

Block copolymers of ethylene glycol and propylene glycol (poloxamers® and pluronics® belong to this family of copolymers), which are amphiphilic macromolecules used as surfactants in addition to being temperature-sensitive, have also been combined with chitosan in the form of sandwiched membranes (chitosan being the inner layer), which have been shown to reduce postoperative tissue adhesions,[9] or as nanoparticles (chitosan/PEO-PPO diblock) proposed for the delivery of proteins and vaccines.[175,176]

The third main water-soluble polymer with known applications in pharmaceutics and medicine that has been used with chitosan is PVA. Some works describe the blending of both materials (although it has been shown that polymers present a low compatibility) intending to modulate the properties of PVA-based hydrogels, which are usually obtained by freeze–thaw cycling.[177,178] Some studies clearly showed that the blends presented improved cytocompatibility (fibroblast adhesion and proliferation) compared with the PVA hydrogels.[179,180] Graft copolymers (chitosan-*g*-PVA) obtained using ceric ammonium nitrate have also been reported.[181] These graft copolymers presented low pH sensitivity (compared with chitosan), and grafting was able to modulate the release of prednisolone as model drug.

10.8 CHITOSAN/SYNTHETIC ANIONIC POLYMERS

Ionized chitosan can form polyelectrolyte complexes (PEC) with (water-soluble) anionic synthetic macromolecules, with polyacrylic acid (PAA) being the most investigated. These types of complexes can be obtained by mixing aqueous solutions,[182] but also by the so-called template polymerization, which implies the polymerization of the anionic monomer in the presence of chitosan[183] (Figure 10.2). Crosslinking of these complexes with glutaraldehyde gives rise to a complex/semi-IPN structure.[182] These structures (crosslinked or uncrosslinked) can exhibit certain pH (at very low or high pH, the complex interaction may disappear) and salt sensitivity. On the other side, thermal treatment of the complex can lead to the grafting or crosslinking by amide formation.[184]

PAA is a very interesting polymer. Trademarks such as Carbopol® are based on this structure. Because it is nontoxic and mucoadhesive, it has been used in some oral pharmaceutical formulations. Chitosan is also mucoadhesive and enhances transmucosal penetration. Stoichiometric complex of both polymers, however, exhibits very poor mucoadhesivity because this is associated with the ionized groups. Therefore, chitosan/PAA formulations for mucosal delivery are actually nonstoichiometric. Usually PAA, which exhibits a better mucoadhesivity, is in excess.[185,186] Release of drugs such as triamcilonone acetonide (frequently used to reduce inflammation in the process of treatment of mouth ulcer) has been evaluated.[187] Crosslinked chitosan microparticles have been grafted with PAA and evaluated for aspirin delivery (into the intestine).[188] If stomach delivery is wanted, some of the chitosan pH sensitivity must be kept (system must expand at acid pH), and formulations must have an excess of chitosan. Amoxicillin freeze-dried hydrogels have been

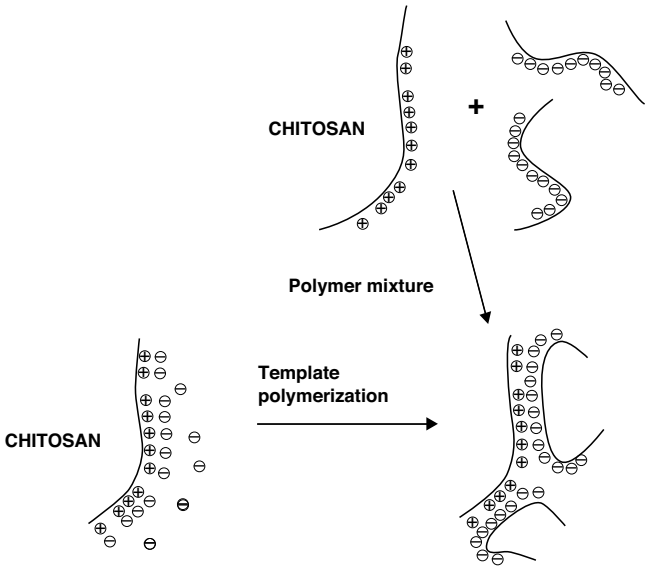


FIGURE 10.2 Scheme of the two possibilities of obtaining chitosan/anionic synthetic polymer PEC.

proposed as suitable matrices for the *Helicobacter pylori* treatment.[189,190] In both situations, the nonstoichiometric modification with the complementary polymer improves certain properties, such as modulated swellability and drug release or lower solubility. Besides mucosal delivery systems, other chitosan/PAA-based drug carriers have been reported, e.g., some pH-sensitive nanoparticles obtained by template polymerization.[191]

In our laboratory, we have prepared chitosan/PAA matrices by template polymerization at low temperature profiting by the activation for the decomposition of the initiator by chitosan.[192] It was also found that during polymerization under these mild conditions, some grafting of polyacrylic acid (PAA) chains onto chitosan molecules occurred, giving rise to weakly crosslinked hydrogels after neutralization. These hydrogels exhibited water uptakes as high as 560 g of water per gram of dry sample. The mechanical properties of these gels were enhanced by copolymerization of acrylic acid with methyl acrylate without loss of the swelling capacity.[193] We have also prepared, by template polymerization, porous CHI/PAA microspheres. They were prepared by a one-step method involving inverse suspension free-radical polymerization of acrylic acid in the presence of chitosan using sunflower oil as the continuous phase. The microspheres have been evaluated as drug delivery systems using meclofenamic acid (MF) as model drug. The swelling of particles is pH dependent; however, the release of meclofenamic acid from particles loaded with the drug is modulated by the water solubility of the drug. Experiments *in vivo* showed that these particles are biocompatible, biodegradable, and resorbed by the living tissue.[194]

Few examples can be found on the complex formation of chitosan with other anionic synthetic polymers, such as polymethacrylic derivative of salicylic acid (which are very interesting polymeric drugs),[195] poly(sodium styrene sulfonate),[196] or poly(2-acryloylamido-2-methylpropanesulfonic acid).[197]

10.9 CHITOSAN/LIPIDS

It is well known that chitosan interacts with the lipids present in the diet or in the cell membranes. In fact, it is used as nutraceutical and has been described as hypocholesteremic. Besides this, some chitosan/lipid systems have been described for the oral delivery of different active compounds. The incorporation of chitosan will affect the interaction with the mucosa as well as other factors such as

the release rate or stability. Chitosan/liposome systems can be obtained by coating preformed vesicles or by adding chitosan to the lipidic bilayer during liposome preparation.[198] Coating of liposomes with chitosan, which has been shown to increase stability[199] and to improve mucoadhesivity,[200] has been described for the delivery of calcitonin (showing an enhanced and prolonged reduction in blood calcium concentration),[201] superoxide dismutase,[202] and for the oral and pulmonary administration of peptide drugs.[129] Thermosensitive chitosan-*b*-glycerophosphate hydrogel containing liposomes has also been proposed for the delivery of hydrophilic molecules.[203] Liposomes encapsulated in alginate chitosan gel capsules have been developed for insulin delivery.[58] Inverse coating, that is, liposome coating of chitosan beads to modulate the drug release, has also been reported.[204]

Alonso et al. have described the preparation of chitosan-coated lipid nanoparticles[205,206] (made from solid lipids) and nanocapsules[207,208] (with an inner oil core) and their evaluation in the delivery of the peptide calcitonin. The coating with chitosan, which can be obtained by mixing the preformed nanoparticles with a polymer solution, improved the stability of the nanoparticles in stomach[209] and enhanced the oral absorption of the peptide calcitonin in calcitonin-loaded nanoparticles. Nasal administration of the calcitonin-loaded nanocapsules reduced significantly the serum calcium level compared with the free peptide solution and nanoemulsions, and the reduced calcium level was sustained for a period of 12 h. This novel system is a promising carrier for improving the nasal absorption of peptide drugs.

Different covalent (lipidic) derivatives of chitosan can also be found in the recent literature. Lauryl derivatives form micelles that can entrap taxol and have been proposed as injectable carriers of this drug.[210] Palmitoyl chitosan-derived vesicles encapsulated in liposomes (vesicle in vesicle system) have also been described as promising drug delivery systems.[211]

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11 Processing and Biomedical Applications of Degradable Polymeric Fibers

K. Tuzlakoglu and Rui L. Reis

CONTENTS

11.1 INTRODUCTION

Fibers are the fundamental units of textile and fabrics. They can be directly supplied from nature or produced from synthetic polymers. Both natural and synthetic fibers have been widely used for biomedical applications.[1] Polymeric fibers that are used in medicine can be manufactured from a wide range of processes, such as melt spinning,[2] dry spinning,[3] and wet spinning.[4]

11.2 PROCESSING OF POLYMERIC FIBERS

11.2.1 Melt Spinning

In melt spinning, polymer granules are melted to a viscosity suitable for extrusion (Figure 11.1). The molten polymer is extruded at high pressure and constant rate through a spinneret to form continuous strands of polymeric fibers. The cooling gases are used in order to solidify the fibers. The lubricants and finishing oils are sometimes applied to the fibers in a spin cell. Then, the fibers are rolled by initial drive roll, which controls the initial take-up speed. The fibers may undergo

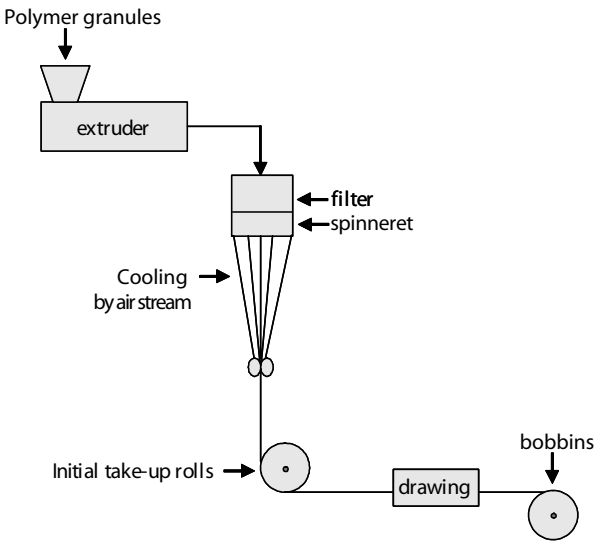


FIGURE 11.1 Schematic diagram of melt-spinning process.

subsequent heating and stretching to achieve molecular orientation. Finally, the fibers are taken up onto bobbins at a constant speed, with a special control device.

Almost all commercially available biodegradable polymers can be processed by a melt-spinning technique.[5,6] In order to prevent hydrolytic degradation, special consideration must be given to the drying of the polymers both before and during processing. Because most biodegradable synthetic polymers are synthesized by ring-opening polymerization, a thermodynamic equilibrium exists between the forward or polymerization reaction and the reverse reaction that results in monomer formation. Excessively high processing temperatures may result in monomer formation during the molding or extrusion process. The excess monomer can act as a plasticizer, changing the material's mechanical properties, and can catalyze the hydrolysis of the material. Therefore, these materials should be processed at the lowest temperatures possible.

11.2.2 Dry Spinning

Although the name of this process is dry spinning, the polymer is certainly wet by a solvent in the beginning of the process. The schematic representation of this process is given in Figure 11.2. In dry spinning, the process starts by dissolving the polymer in an organic solvent. Some additives can be blended with the polymer solution in this stage. This viscous polymer solution is called "spin dope" for spinning. Then, the fibers are formed by extrusion of polymer solution through tiny holes in a spinneret plate. The heated air or gas stream into the spinneret zone leads to the removal of the solvent from the filaments. Finally, the fibers are taken up to the rolls as in melt spinning.

Both melt- and dry-spinning processes are used for the production of commercially available biodegradable fibers from polylactide, polyglycolide, polycaprolactone, and their copolymers. For instance, the production of polylactide (PLLA) fibers by melt and dry spinning has been extensively studied. PLLA fibers with different tensile strengths such as 0.53 GPa,[7] 0.87 GPa,[2] and 2.4 GPa[5] have been produced by melt spinning, sometimes following hot drawing process. Higher-strength PLLA fibers can be obtained by dry spinning. Eling et al.[8] produced PLLA fibers with tensile strength 1.0 GPa by dry spinning, while it was 0.5 GPa for melt-spun fibers. They have also produced high-strength PLLA fibers with 2.1 GPa tenacity by dry spinning and hot drawing of PLLA from solutions in chloroform/toluene mixtures[3] and studied the effect of drawing conditions on dry-spun PLLA fibers.[9] However, dry spinning is not easy to control and is typically more

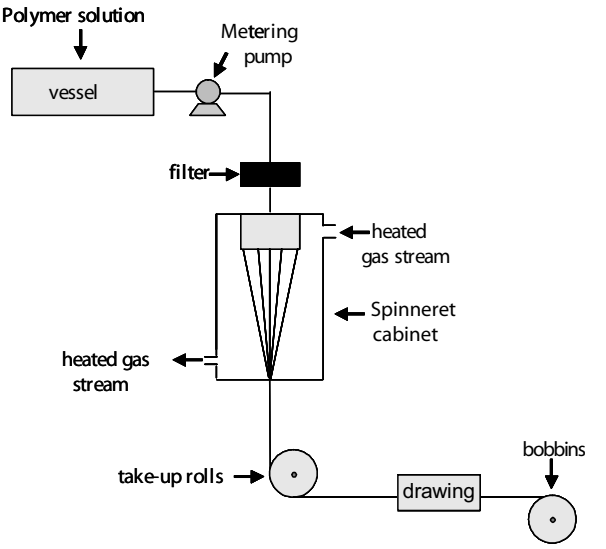


FIGURE 11.2 Schematic diagram of dry-spinning process.

expensive than melt spinning. Therefore, melt spinning is still preferred for production of PLLA fibers. In the case of polyglycolide fibers, dry spinning is not preferred due to the intractable nature of the polymer, which makes PGA fibers have lower strength than PLLA fibers. Fibers from copolymers of *L*-lactide with glycolide, *D*-lactide, and ε-caprolactone can be also produced by either melt spinning or dry spinning.[7,10]

The melt-spinning method has been also used to process biodegradable bacterial poly(3-hydroxybutyrate) (PHB) and PHB copolymer fibers, which can be used in different tissue engineering applications.[6,11] Recently, another new biodegradable based on ε-caprolactone and 11-aminoundecanoic acid has been formed in fibers by melt spinning.[12] Depending on the monomer ratios, these new polyesteramide fibers can have 102–140 MPa tensile strength.[12]

11.2.3 Wet Spinning

Wet spinning is the oldest method of fiber spinning (Figure 11.3). As in dry spinning, the polymer is dissolved in a suitable solvent. However, after passing through the spinneret, the polymer enters a coagulation bath. Either the bath reacts chemically to coagulate the polymer, or it draws out the

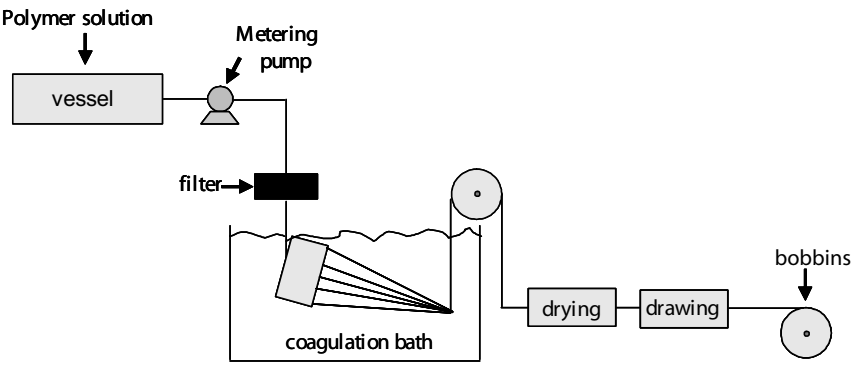


FIGURE 11.3 Schematic diagram of wet-spinning process.

solvent from the polymer stream so that the filament can harden. In most cases, the second liquid is aqueous. A main difference between wet spinning and either melt or dry spinning is that one is spinning into a fluid with a much higher viscosity. Due to this higher viscosity, higher shearing stress occurs on fiber surfaces, which introduces very high tension into the filaments.

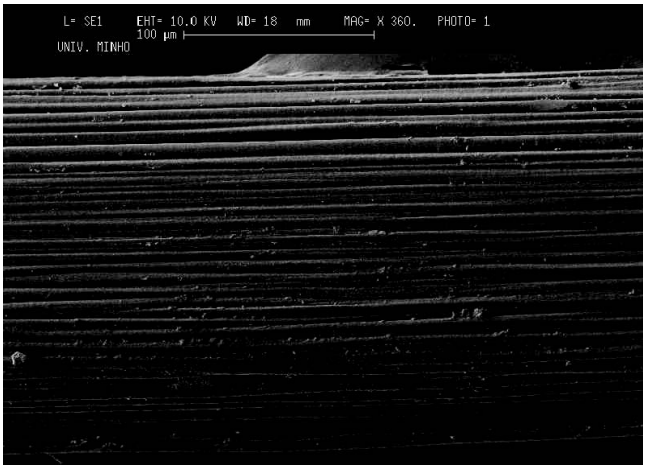
Please note that the extrusion may be carried out directly into the coagulating liquid or through a small air gap. In the second case, it may be known as dry-jet wet spinning or air-gap wet spinning.

Wet spinning is mostly used to produce natural fibers, such as chitin and chitosan fibers, that cannot be formed by either melt- or dry-spinning methods. The strong inter-chain forces as derived from the hydroxyl, acetamido, and amino groups raise the melting point of chitin and chitosan to well above their thermal degradation temperatures. Therefore, melt spinning is typically not possible for chitin and chitosan.[4] Besides that, these two natural polymers can only be dissolved in polar solvents that have high boiling points. As a consequence, dry spinning is also not practical for producing chitin and chitosan fibers. In the case of chitin fibers, a number of solvent–coagulant systems have been tried to form the chitin fibers. For instance, a chitin solution in LiCl-*N,N*-dimethylformamide has been spun into *n*-butanol,[13] each of sodium chitin xanthate and sodium chitin salt solutions in aqueous NaOH have been spun into an aqueous H_2SO4 solution containing Na_2SO4 and ZnSO4,[14] and a chitin solution in formic acid–dicholoroacetic acid has been spun into acetone.[15] Spinnability of the chitin fibers can be improved with increasing coagulation bath temperature and with addition of the right type of plasticizer.[4] Wet spinning of chitosan fibers is obtained by extruding the viscous chitosan solution in dilute acid into a coagulation bath. The coagulation bath must exhibit a high pH, such as aq. NaOH,[16] aq. KOH,[17] aq. NaOH–40% methanol,[18] and aq. NaOH–$NaSO_4$ (or AcONa) mixture.[19] In order to obtain fibers with good mechanical properties, some physical and chemical treatments, which are called drying treatments,[20] can be used at the end of the process. It has been found that the drying treatment, as well as spinning conditions, have a strong effect on the fiber properties.[17,20] Figure 11.4 presents wet-spun chitosan fibers with diameter of 200 μm, which have been prepared using methanol drying treatment at the end of the processing.[21] Recently, chitosan–collagen blend fibers have been produced by means of a wet-spinning method in order to improve the blood compatibility of chitosan fibers.[22] Moreover, chitosan-coated alginate filaments have also been prepared using a wet-spinning approach aiming at wound healing applications.[23]

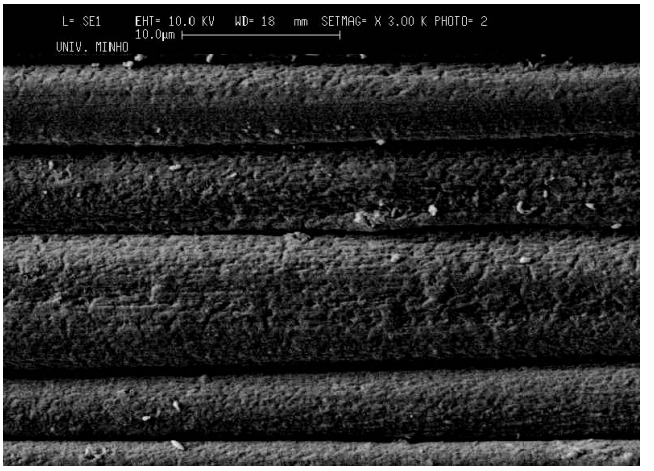
As mentioned previously, the high temperature used in melt spinning causes the thermal degradation. It can be avoided in dry and wet spinning. However, in these processes, residual solvent must be removed efficiently in order to prevent toxicity or other problems in medical applications. They are therefore more expensive than melt spinning. Furthermore, in the case of dry spinning, rough fiber surface is obtained, while the fiber surface is smooth in melt spinning. The rougher surfaces might be preferred for some applications.

Besides those three main spinning methods, there are some other special methods in fiber processing. One of these methods is gel spinning, which is used to obtain high strength or other special fiber properties. In gel spinning, the polymer in gel phase is extruded as in dry spinning. Gel form of the polymer produces strong inter-chain forces in the filaments that can significantly increase the tensile strength of the fibers. Moreover, in gel phase the polymer chains are bound together at various points in liquid crystal form. These liquid crystals are aligned along the fiber axis by shear forces during extrusion and cause highly oriented fibers. For instance, recently oriented elastic fibers of poly(β-hydroxybutyrate) (PHB) with 360 MPa tensile strength and 5.6 GPa of modulus have been produced by a gel-spinning technique.[24]

Another new technique in fiber production is called electrospinning, which can produce fibers with submicron diameters (Figure 11.5). The principle of electrospinning is to use an electric field to draw a positively charged polymer solution from an orifice to a collector. This electrical field between capillary tip and the collector induces a charge on the surface of the polymer solution or melt. As the electrical field is increased, the hemispherical shape of the drop at the end of the capillary tip is changed to a conical shape known as the Taylor cone.[25] When the critical value of



(a)



(b)

FIGURE 11.4 SEM micrograph of chitosan fibers: (a) ×360 magnification, (b) ×3000 magnification.

electrical field is exceeded, a charged jet of the solution is ejected from the tip of Taylor cone. As the jets moves toward a collecting metal screen, solvent evaporates and solidified fibers are collected on the metal screen.

Electrospun fibers can be easily formed into 3-D structures during their deposition.[26] Furthermore, their small pore size and large surface area make them suitable candidates for biomedical applications such as tissue engineering scaffolds. In recent years, biodegradable electrospun fibers, such as poly-*L*-lactide, poly(*D,L*-lactide), and polycaprolactone, have been studied by several researchers for potential use in biomedical applications.[27,28]

11.3 FIBER STRUCTURES

Fibers can be formed in 3-D structures such as knitted, braided, woven, and nonwoven. The orientation of fibers into these structures may range from highly regular to completely random. The final structure of the fibers affects the behaviors of the fibers when they are applied. For example, woven structures show more stable and porous structure than the other fiber structures.

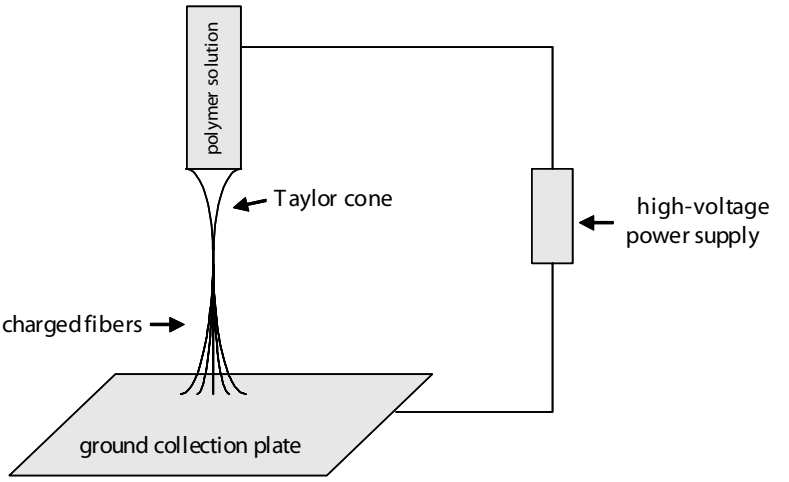


FIGURE 11.5 Schematic diagram of electrospinning process.

As a disadvantage, they can be unraveled at the edges when cut squarely or obliquely for implantation. However, knitted structures have inherent ability to resist unraveling when cut. Moreover, they are very flexible and porous, but sometimes their flexibility is reduced when the additional yarns are used to interlock the loops in order to obtain more stable structures. Additionally, difficulties of reducing their high porosity below a certain value is another clear disadvantage for some applications.

Braided structures are mostly used as a suture.[29] They can be designed using several different patterns, either with or without core. The spaces between the yarns, which cross each other, make them porous and help the fluid flow during the healing process.

Nonwoven structures may have a wide range of porosity. Their isotropic structure provides good mechanical and thermal stability. They can easily compress and expand. These advantages make them a suitable material for many tissue engineering applications.

11.4 MEDICAL APPLICATIONS OF BIODEGRADABLE FIBERS

Fibers that are used in medicine can be called *biotextiles.* A biotextile is defined as a structure composed of textile fibers and designed for use in a specific biological environment, where its performance depends on its interactions with cells and biological fluids as measured in terms of its biocompatibility and biostability.[30] Biotextiles can be nonbiodegradable[31] or biodegradable.[21,28]

In the case of biodegradable polymeric fibers, they have a number of applications in medicine, including sutures, vascular grafts, artificial skin substitutes, ligaments, bone and cartilage scaffolds, and nerve guides, among others.

11.4.1 SUTURES

The oldest example of a biomedical textile is a suture. Sutures are used to repair damaged tissues, cut vessels, and surgical incisions. There are two major classes of sutures: absorbable (or biodegradable), and nonabsorbable (or nonbiodegradable or very slowly biodegradable). Biodegradable sutures are mainly used for healing internal wounds to avoid secondary surgery. The important requirements for the ideal biodegradable suture are (1) absorbability and absorption rate, (2) low toxicity, (3) high tensile strength, (4) knot strength and knot-holding quality, (5) elongation at body conditions and diameter, and (6) sterilizability.

Biodegradable sutures can be manufactured in several forms, namely as monofilament, multifilament, or braided structures. All types have advantages and disadvantages. Monofilament sutures are easy in knot throw-down and less risky in infection, while braided sutures are more susceptible to bacterial invasion. Multifilament sutures also have small risk of infection, but their handling properties are very good.

Silk is one of the first suture materials to be used in surgery. Silks are generally defined as protein polymers that are spun into fibers by some lepidoptera larvae such as silkworms, spiders, scorpions, mites, and flies.[32] Silk is slowly absorbed *in vivo*. The rate of absorption is dependent on the implantation site, mechanical environment, type of source, and the diameter of the fiber. As a suture, silk is still popular in ocular, neural, and cardiovascular surgery and has also been used in a variety of the other tissues in the body.

Biodegradable polyesters fibers derived from aliphatic hydroxycarbolic acids, especially glycolic and lactide acid fibers, are commercially used for this application. Dexon® (polyglycolide; multifilament), Dexon Plus® (polyglycolide, braided), Vicryl® (poly[glycolide-*L*-lactide]; multifilament), Maxon® (poly[glycolide-co-trimethylene carbonate]; monofilament), Biosyn® (poly[glycolide-co-*L*-lactide-co-trimethylene carbonate]), and PDS® (poly-*p*-dioxanone; monofilament) are some examples for commercially available biodegradable sutures.[33] In one comparative study, the degradation rates for Dexon Plus, Vicryl, Maxon, and PDS have been compared, and it was found that the *in vivo* half-life tensile strength of the Dexon and Vicryl is 2 weeks, whereas the half-lives of Maxon and PDS are 3 and 6 weeks, respectively.[33] Additionally, Maxon demonstrated the best *in vitro* knot security.

A monofilament suture made of poly(lactide-co-caprolactone) (P[LA/CL]) has been also suggested as a biodegradable suture.[34] It showed almost similar knot-pull strength to PDS and Maxon, although the tensile strength of P(LA/CL) suture is lower. *In vivo* studies have showed that P(LA/CL) caused very mild tissue reaction, similar to PDS.[34]

More recently, Boccaccini et al.[35] developed a bioactive suture by coating Vicryl suture with Bioglass® particles for applications in wound healing, fabrication of fibrous three-dimensional scaffolds for tissue engineering, and reinforcement elements for calcium phosphate temporary elements.

Polyhydroxyalconate polymeric fibers, which are biodegradable and biocompatible natural fibers, have also been investigated for use as a suture.[36] Monofilament sutures made of poly-3-hydroxybutyrate (PHB) and poly-3-hydroxybutyrate-co-poly-3-hydroxyvalerate (PHB/PHV) have shown no adverse effect *in vivo* and had enough strength during the healing period of the wound.

11.4.2 Wound Healing

Another application of biodegradable polymeric fibers is wound dressing. Natural fibers, such as chitin, chitosan, and a benzyl ester of hyaluronic acid (Hyaff 11®), are mainly used for wound healing due to their wound healing ability. Hirano et al.[37] developed a new biocompatible dressing material made of wet-spun chitin–acid glycosaminoglycan fibers that released a portion of the glycosaminoglycan in the body. Hyaff 11-based materials have been also studied for wound healing.[38,39] This material showed good ability to treat deeper lesions where there is great loss of dermal tissue.

11.4.3 Ligament Tissue Engineering

Ligaments are bands or sheets of fibrous connective tissue connecting two or more bones. The role of the ligament is to augment the mechanical stability of the joints, to guide the joint motion, and to prevent excessive motion. The ligament consists of approximately 77% (dry weight) collagen and less than 1% elastin, with the proteoglycans and glycoproteins making up the rest.[40] The dimensions of the human anterior cruciate ligament (ACL) range from roughly 30 to 38 mm long and 10 to 13 mm wide. The ultimate tensile strength of the human ACL is approximately 1730

N.[41] Ligaments, in general, are collagen fiber bundles, which are grouped into larger bundles, of specific orientation. Due to this structure of ligament tissue, collagen fibers are widely used for ligament replacements. Gentleman et al.[42] extruded bovine Achilles tendon collagen fibers with diameter of 125 μm, which showed similar mechanical properties to human ligaments. Silk is another natural polymer that has been proposed for tissue engineering of anterior cruciate ligaments.[43] When they are properly prepared, silk fiber matrices, which have good mechanical properties as well as biocompatibility and slow degradability,[43] can provide suitable matrices for the support of adult stem cell differentiation toward ligament lineages. Another available material for ACL is polydioxanone fibers (PDS). The fast degradation of PDS, however, could be a problem for this application.[44] The half-life tensile strength of PDS is only 4–6 weeks, whereas the process of revitalization and recovery of the transplanted tendon graft can take up to 12 months.

Alternative degradable materials in anterior cruciate ligament reconstruction are the synthetic polyester poly-*L*-lactide, polyglycolide, and its copolymers.[45–47] They have mainly been used to reinforce the primary suture of the mid-part tears of the anterior cruciate ligament. Cabaud et al.[45] used braided polyglycolide ligaments, which showed 828 N max. linear load at 22.6%, for the repair of anterior cruciate ligaments of dogs. Although they showed high initial strength, degradation time was not long enough to protect the repaired ligament. Poly-*L*-lactide fibers in braided form have been tested for the augmentation of ACL ruptures in rabbits and showed better *in vivo* results than polyglycolide and PDS.[46] In order to obtain a material with the suitable degradation rate for ACL, Durselen et al.[47] investigated the degradation kinetics of a variety of different degradable fibers made of poly-*L*-lactide and poly(*L*-lactide-co-glycolide). It was found that the strength of the fiber braids decreases much faster than their stiffness, which makes their application as a ligament augmentation construct. However, this problem could be solved by designing an augmentation construct braided out of multiple fibers with variable degradation rates.

11.4.4 Cardiovascular System Applications

Biomedical textiles are widely and successfully used as vascular prostheses, including high-flow-rate arteries.[48–50] Most are woven, knitted, or microporous tubular structures that are supple and resemble the softness and flexibility of natural blood vessels. However, these porous prostheses must be plugged in order to prevent blood loss after implantation. This is normally accomplished either by preclotting in blood before implantation or by allowing the clot to form *in situ*. Nonbiodegradable synthetic polymeric fiber structures, such as Dacron and Teflon, are used as vascular grafts.[51] Woven structures made of biodegradable polymers were applied as a temporary scaffold for the regeneration of the arterial wall. A fully biodegradable vascular graft made from a knitted mesh of polyglactin 910 (Vicryl) sheets was investigated in 1979.[48] *In vivo* animal experiments showed endothelization of the luminal surface and formation of multiple layers of the smooth muscle cell layers that lacked elastic laminae, which was ascribed to compliance mismatch of these prostheses. Similar results were found with the woven tubes of polyglycolide (PGA) and polydioxanone (PDO) as a replacement part of the intrarenal aorta in rabbits.[49] The PGA component of the woven grafts was totally resorbed within 6 months, while the PDA component was resorbed within 6 months. The regenerated arteries withstood 800 mmHg of pulsatile systolic pressure *ex vivo* without bursting. Shum-Tim et al.[50] used nonwoven PGA mesh combined with biodegradable polyhydroxyalkanoate (PHA) to create a new vascular autograft for use in an aortic position.

After some initial attempts at directly implanting biodegradable grafts that are totally dependent on tissue ingrowth *in vivo*, cell seeding onto biodegradable scaffolds has been provided to initiate functional tissue regeneration. Smooth muscle cells (SMCs) that were seeded onto PGA fiber-based matrices have shown high cellular viability and produced large amounts of extracellular matrix proteins.[52] Recently, Xu et al.[53] suggested that nanofibrous aligned poly(*L*-lactide-co-ε-caprolactone) scaffolds produced by electrospinning could be used for blood vessel engineering. SMCs showed good attachment and migration along the axis of the aligned nanofibers and expressed a

spindle-like contractile phenotype. Moreover, it has been found that distribution and organization of smooth muscle cytoskeleton proteins inside SMCs were parallel to the direction of the nanofibers.

The other application of biodegradable polymeric fibers in the cardiovascular field is the tissue engineering of heart valves. The clinically used prosthetic heart valves include xenograft valve, mechanical valve, and homograft valve. There are limitations to the long-term benefits of these valve prostheses, however, such as poor durability, foreign body reaction, infection, anticoagulation problems, or donor scarcity. Recently, many studies[54–57] have been undertaken to determine if tissue engineering principles could be used to develop valve tissue substitutes. Seeding of human fibroblasts on biodegradable fiber mesh is a new approach for creation of the human autologous tissue-engineered heart valves. Polyglycolide (PGA) nonwoven scaffolds are the most-used materials for this purpose.[54,55] In order to improve cell attachment and proliferation, PHB-coated PGA and PLA nonwoven fiber mesh structures have also been studied under dynamical conditions for developing a tissue-engineered heart valve.[56,57]

Biodegradable fiber structures also have been proposed for use as a stent.[58–61] Stents mechanically support vessels against elastic recoil, thereby reducing the restenosis rate in percutaneous coronary interventions. Biodegradable stents are optimal if the need for stenting the airway is only temporary. For instance, Paclitaxel-loaded melt-extruded poly-*L*-lactide fibers have been tried as a stent.[59] Furthermore, Saito et al.[58] studied a biodegradable knitted stent made of poly-*L*-lactide in rabbit airways. The results have shown that the knitted tubular PLLA stents could be used like commercially available silicone stents. Nuutinen et al.[60] developed a biodegradable stent from PLLA fibers using a braiding technique. These stents showed radial pressure stiffness similar to commercial metallic stents. They have also studied the mechanical properties and *in vitro* degradation of biodegradable knitted stents made of polylactide, poly(*L*-lactide-co-*D,L*-lactide) and poly(*L*-lactide-co-glycolide) fibers.[61] It has been shown that different chemical composition of these fiber structures provided different degradation rates, which can be tailored for specific applications.

11.4.5 Bone and Cartilage Tissue Engineering Applications

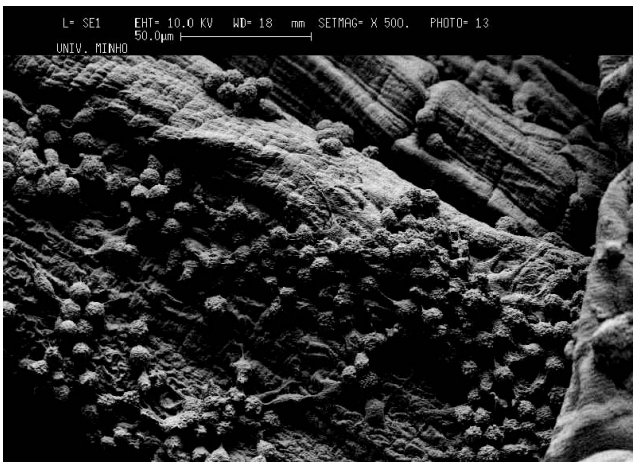
Biodegradable knitted, woven, and nonwoven structures have shown promise for use as tissue engineering scaffolds for seeding of chondrocytes[62,63] and osteoblasts.[21] Their structures provide a large surface area and a relatively large porosity, which can be optimized for specific applications. To design a scaffold for bone and cartilage tissue engineering, many parameters must be considered, such as optimal fiber diameter and linear density, overall porosity and pore size distribution, influence of fiber orientation on cellular response, and influence of degradation on the properties of the structures. It has been shown that the cellular response to the polymeric fibers was directly dependent on fiber diameter, with finer fibers generating thinner surrounding tissue capsules *in vivo*.[64] Polymeric fiber structures made by poly-α-hydroxy acids are widely used to engineer the cartilage and bone tissues. Ma and Langer[62] attempted to engineer the cartilage tissue by seeding articular chondrocytes onto polyglycolic acid nonwoven scaffolds. They reported that the aggregate modulus of the engineered cartilage reached 179 + 9 kPa after 20 weeks of *in vitro* cultivation, which was 40% that of natural articular cartilage. For tissue engineering cartilage, nonwoven polylactide-co-glycolide mesh structures can be coated by alginate to provide the ability of the cells to suspend and to distribute cells within the polymeric mesh.[63] Alginate also stimulates the chondrogenic phenotype of the transplanted chondrocytes and prevents cells from floating out of the defects. Using the same approach, Ameer, Mahmood, and Langer[65] have prepared a composite, which was composed of a polyglycolide nonwoven mesh coated by fibrin gel, for use in meniscal surgery. At 4 weeks in culture, glycosaminoglycan (GAG) content in fibrin-coated PGA mesh scaffolds was better than the uncoated PGA mesh scaffolds. These new approaches have promised to generate structurally regular cartilage.

More recently, hyaluronic acid-based nonwoven scaffolds (Hyaff 11) have been used in bone and cartilage regeneration.[66,67] In order to obtain a nonwoven mesh structure, hyaluronan total

benzyl ester thread is produced by phase separation extrusion technology and then cut, carded, and needle-punched. Radice et al.[66] reported that Hyaff 11 nonwoven scaffolds were a suitable support for mesenchymal progenitor cells, which produced the main extracellular matrix molecules, accompanied by an occasional synthesis of mature type II collagen. Moreover, when they were implanted, with or without cells, there was no inflammatory response and they degraded within 4 months after implantation. In addition, nonwoven Hyaff 11 scaffolds promote mineralization of rat bone marrow stromal cells and enhance it in the presence of basic fibroblast growth factor (bFGF).[67]

Chitosan-based nonwoven structures have been also proposed as bone and tissue engineering scaffolds.[21,68] Tuzlakoglu et al.[21] reported that osteoblast-like cells growing over chitosan nonwoven scaffold surfaces presented adequate morphology and good proliferation after 7 days of culture (Figure 11.6).

(a)



(b)

FIGURE 11.6 Osteoblast-like cells proliferating over chitosan-based fibers after 7 days of culture: (a) ×100 magnification, (b) ×500 magnification.

Finally, within the last few years, biodegradable nonwoven nanofibers, which are produced by electrospinning, have been used as scaffolds for regeneration of cartilage and bone. The rationale for using nanofibers is based on the theory that cells attach and organize well around fibers with diameter smaller than the diameter of the cells. Three-dimensional matrices made of polycaprolactone nanofibers with diameter of 700 nm have been proposed as a scaffold for cartilage tissue engineering.[27] The primary chondrocytes that were seeded onto these scaffolds proliferated and efficiently maintained their differentiated phenotype, as indicated by the expression of cartilage-associated genes. Nonwoven polycaprolactone nanofibers have been also tried with mesenchymal stem cells derived from the bone marrow of neonatal rats under dynamic culture conditions.[28] After 4 weeks of culture, it has been found that the surfaces of the cell–polymer constructs were covered with cell multilayers. Additionally, mineralization and type I collagen were observed at 4 weeks.

Besides those applications, biodegradable polymeric fibers have found uses in dental applications as a guided tissue regeneration (GTR) membrane.[69,70] These barrier membranes prevent epithelial migration and promote the regeneration of new connective tissue attachment. Vicryl periodontal mesh and Gore Resolut® (composed of polyglycolide fiber and trimethylene carbonate) are successfully used for this application.[69] PLA-coated knitted PGA mesh has been also studied for use as a barrier membrane in GTR.[70]

Both natural and synthetic biodegradable polymers have also been used for guided nerve regeneration. For instance, a nerve guide made of collagen filaments has been proposed as an alternative to the tube-type nerve conduits.[71] The collagen conduits that consist of 2000 collagen filaments showed better regeneration than the collagen tubes, which were used as a control, in regeneration of rat static nerve. Synthetic biodegradable fibers made of poly-*L*-lactide have been examined using dorsal root ganglia *in vitro*.[72] The results have demonstrated that PLLA filaments orient the growth of Schwann cells and neuritis along the longitudinal axis of the filament. In addition, PLLA fibers coated with laminin or Schwann cells have also been used for guided nerve regeneration.[72] Recently, Cheng and Chen reported that Schwann cells were able to migrate and proliferate on the polyglactin 910 (Vicryl) fiber.[73]

11.5 CONCLUSIONS AND FUTURE ASPECTS

Nowadays, medical devices and surgical products constructed from degradable fibers are generating much interest and are starting to be commercialized. This interest is mainly due to the structural properties of the fibers, which allow them to be formed as a different product depending on the application. Development of new materials and production techniques and the improvement of existing materials and techniques will influence the uses of degradable fibers in the biomedical field. However, there are many parameters that need to be addressed when a new material or technique is designed for medical applications. Fiber size, surface topography, degradation rate, adequate mechanical strength, three-dimensional structure, porosity, and permeability must be suitable for the desired application. Additionally, developments in fiber technology will help introduce new products to the biomedical market.

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12 Understanding the Enzymatic Degradation of Biodegradable Polymers and Strategies to Control Their Degradation Rate

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CONTENTS

12.1 INTRODUCTION

Nature has always provided, in considerable amounts and variety, polymeric materials with interesting compositions and structures and with great potential to be used in biomedical applications. These natural polymers include polysaccharides,[1] such as cellulose, chitin, starch, alginate, galactan, hyaluronic acid, dextran, and gellan, obtained from plants, animals, and microbial sources and polyesters,[2] such as poly(β-hydroxybutyrate) and poly(β-hydroxybutyrate-co-β-hydroxyvalerate), produced by numerous bacteria. In biological systems, polysaccharides and their derivatives are found as energy reservoirs, as components of the cell wall of plants, in bacteria, and in the connective tissues.[3,4]

Since the early 1960s, several synthetic degradable polymer systems have been used as medical implant materials,[5] which include various polyesters, polyurethanes, polyanhydrides, polyacrylates, polyphosphoesters, and polydiaxanone, among others.[3,6] Over decades of research, many new biomaterials have been developed, but the prospect for novel biomaterials and novel applications continues to be immense.

Applications of biodegradable polymers in medicine (please see Chapter 1 by Suzuki and Ikada in this book) include resorbable surgical sutures, matrices for the controlled release of drugs, scaffolds for tissue engineering, and resorbable orthopedic devices such as bone cements, pins, screws, and plates.[7] The development of biomaterials requires an extensive evaluation, in terms of biocompatibility, mechanical properties, and degradation behavior, in order to determine whether a certain material is suitable for a particular application. Understanding the factors that control the degradation of biomaterials is, therefore, critical for the development of degradable polymeric systems, and a greater comprehension of these mechanisms would be helpful to optimize their current usage. The demand for biomaterials with controlled, predictable degradation kinetics includes a wide range of biomedical applications, and this had led to extensive research on the degradation behavior of a variety of biodegradable polymers. Great attention has been devoted to the study of degradation mechanisms of poly(lactic acid)-based polymeric systems, and a vast literature (research articles[8–15] and reviews[6,16]) is now available.

This chapter intends to provide the reader with an overview on the degradation mechanisms of biodegradable polymers with special emphasis on the main parameters affecting the enzymatic degradation of polymeric biomaterials. For that, a range of potential enzymes involved in the degradation of polymeric biomaterials will be considered, taking into account their normal levels in the human body fluids and their secretion during inflammatory responses. Some guidelines will be given for designing *in vitro* degradation studies, including a detailed description of the main characterization techniques that should be used to evaluate the degradation behavior of biomaterials under specific conditions. At the end of the chapter, a practical study about the enzymatic degradation of starch-based polymers will be presented and discussed, as well as a case study that was aimed to assess the degradation behavior of starch-based polymeric biomaterials under the influence of certain enzyme activities. In addition, the effect of α-amylase encapsulation in starch-based matrices on their degradation profile was studied and will be presented herein with the perspective of developing biomaterials with enzymatically controlled degradation rates.

12.2 IMPORTANCE OF BIODEGRADABILITY IN BIOMEDICAL APPLICATIONS

The term *biodegradation* is often used to denote degradation occurring in a biological environment. In the context of biomedical applications, biodegradation may be defined as the "gradual breakdown of a material mediated by a specific biological activity."[17]

The performance of medical devices depends largely on the stability of the material, and biodegradation is a key issue on the list of safety standards when choosing materials for biomedical applications.

Biodegradable polymers are materials with the ability to function for a temporary period and subsequently degrade, under a controlled mechanism, into products easily eliminated in the body's metabolic pathways. In this way, biodegradability not only eliminates the risk of complications associated with the long-term presence of a foreign material and the need for a second surgery for implant removal, but also allows for improved healing, as viable tissue interacts and grows into the degrading construct.

The use of polymeric materials in drug delivery applications also requires that the polymer degrade under physiological conditions and slowly release the encapsulated drug. The polymer should demonstrate, therefore, a continuous mass loss profile to facilitate repeated dosing and to ensure the successful effect of the treatments.

The degradation of biomaterials is also important in terms of biocompatibility, since the changes that occur in the physicochemical properties of the materials during degradation may alter their functionality and the associated biological response. In addition, the nature of the degradation products will, in part, define the ultimate biocompatibility of the materials since it may also induce alterations to cellular function.

Understanding the degradation mechanisms of biomaterials (degradation kinetics, evolution of mechanical properties, identification of degradation products) is, therefore, of crucial importance when selecting and designing materials for specific applications since the degradation process may affect a range of events such as cell growth, tissue regeneration, drug release, host response, and the material function.

12.3 DEGRADATION PROCESSES IN BIODEGRADABLE POLYMERS

The degradation of a biomaterial can occur at different stages of its preparation, including during its storage. For instance, it was observed that the molecular weight of poly(*L*-lactide) decreased from 431,000 to 202,000 Da upon storage.[8]

The conditions used during the processing and fabrication of polymeric materials may also lead to polymer degradation, consequently influencing their degradation behavior *in vivo*. Melt-based techniques (injection molding, extrusion, compression molding) are performed at high temperatures and in the first two cases at high shear rates, which may cause some degradation of the material. The production of samples by injection molding leads to a partial material orientation, which is typically higher in the skin than in the core of the molding. The chain orientation across the sample upon processing may be responsible for a faster degradation in the center than in the skin.[8] In addition, it is very common to use additives (plasticizers, lubricants, antioxidants, salts, stabilizers) during the processing of polymeric materials, which will leach out after immersion and may enhance or inhibit the degradation process.

Another aspect to be considered is the fact that biomedical materials need sterilization before being implanted. Sterilization can be performed using heat, steam, gas (ethylene oxide, EtO), or ionizing radiation, mainly γ or β. Each of these sterilization methods may have an effect on the material degradation, but sterilization by radiation requires high doses of high-energy radiation, resulting in some cases in polymer crosslinking and degradation. γ sterilization was shown to reduce significantly the molecular weight of poly(lactide–glycolide) polymers,[6] but cold-cycle EtOH sterilization did not cause any changes in the molecular weight of polylactides.[8] Mechanical stress may also affect the degradation, either as a result of loading under service or due to residual stress arising during manufacturing, but this type of degradation is more significant on materials subjected to mechanical stress such as sutures, scaffolds for tissue engineering, and fixation devices.[6]

Materials exposed to the body fluids may undergo changes in their physicochemical properties as a result of chemical, physical, mechanical, and biological interactions between the material and the surrounding environment. Although materials can be degraded by thermal and mechanical processes, only degradation by oxidation and hydrolysis will be discussed in more detail in the following sections of the present chapter.

12.3.1 Chemical and Enzymatic Oxidation

Polymeric biomaterials may be degraded by chemical and enzymatic oxidation when exposed to the body fluids and tissues. It is well known that during inflammatory response to foreign materials, inflammatory cells, particularly leukocytes and macrophages, are able to produce highly reactive oxygen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), and hypochlorous acid (HOCl).[17–19] The oxidative effect of these species may cause polymer chain scission and contribute to their degradation.

Several studies have been carried out to assess the effect of oxygen species in the degradation of polymeric biomaterials.[17,19,20] Lee and Chu[20] studied the role of superoxide in the degradation of absorbable sutures and found that O_2^- could accelerate the degradation of aliphatic polyesters by the cleavage of ester bonds via nucleophilic attack of O_2^-.

The effect of oxidative enzymes, such as horseradish peroxidase, catalase, and xanthine oxidase, on the degradation of poly(urethane)s (PUs) was also studied, but these oxidative systems were unable to induce degradation of PUs.[21,22]

A more detailed description about how oxidative enzymes, free radicals, superoxides, and peroxides influence the degradation of biomedical polymers can be found in a review by Williams and Zhong.[17]

12.3.2 Nonenzymatic Hydrolysis

Polymer hydrolytic degradation may be defined as the scission of chemical bonds in the polymer backbone by the attack of water to form oligomers and finally monomers. In the first step, water contacts the water-labile bond, by either direct access to the polymer surface or by imbibition into the polymer matrix followed by bond hydrolysis. Hydrolysis reactions may be catalyzed by acids, bases, salts, or enzymes.[17]

After implantation, the biomaterial absorbs water and swells, and degradation will progress from the exterior of the material toward its interior.

The hydrophilic and hydrophobic nature of polymeric materials influences their degradation rate, and the susceptibility to hydrolysis follows this order[17]: (1) hydrophilic with hydrolysable bonds, (2) hydrophobic with hydrolysable bonds, (3) hydrophilic with no hydrolysable bonds, and (4) hydrophobic with no hydrolysable bonds. For instances, *N*-vinylpyrrolidinone (NVP) is capable of absorbing relatively large amounts of water, but it is not prone to hydrolysis.[23]

All biodegradable polymers contain hydrolysable bonds, such as glycosides, esters, orthoesters, anhydrides, carbonates, amides, urethanes, ureas, etc.[3,6,17] Polymers with strong covalent bonds in the backbone (like C-C) and with no hydrolysable groups require longer times to degrade.[3,24]

12.3.3 Enzyme-Catalyzed Hydrolysis

Enzymes are biological catalysts, i.e., they accelerate the reaction rates in living organisms without undergoing themselves any permanent change. In fact, in the absence of enzymes, most of the reactions of cellular metabolism would not occur. Hydrolysis reactions may be catalyzed by enzymes known as hydrolases, which include proteases, esterases, glycosidases, and phosphatases, among others. This class of enzymes comprises cell-derived proteins that are responsible for the catalysis of several reactions in the human body. For example, hydrolytic enzymes are present in the plasma and interstitium, in the brush border membrane and lumen of the gastrointestinal tract, and in the tubular epithelium of the kidneys, where they ensure the efficient hydrolysis of different substrates to facilitate absorption of nutrients and solutes.[4]

In this sense, it is expected that some of these enzymes may play an important role in the degradation of biomaterials by catalyzing their hydrolysis. It has been shown that the degree of biodegradation of polyurethanes, in the presence of cholesterol esterase enzyme, is about 10 times

higher than in the presence of buffer alone.[21] This fact may explain the higher *in vivo* degradation rates of some biomaterials when compared with *in vitro* experiments.

While some enzymes catalyze only one reaction involving only certain substrates, others are not very specific. This indicates that the degradation of synthetic polymers may also occur by enzymatic hydrolysis, and some examples of this will be given in Section 12.3.3.2.

12.3.3.1 Factors Affecting Enzymatic Hydrolysis

The enzymatic hydrolysis of polymeric biomaterials is a heterogeneous process that is affected by the mode of interaction between the enzymes and the polymeric chains and involves typically four steps[25]: (1) diffusion of the enzyme from the bulk solution to the solid surface, (2) adsorption of the enzyme on the substrate, resulting in the formation of the enzyme–substrate complex, (3) catalysis of the hydrolysis reaction, and (4) diffusion of the soluble degradation products from the solid substrate to the solution. The rate of the global reaction is controlled by the slowest step.

The adsorption and rate of hydrolysis reaction is affected by the physicochemical properties of the substrate (molecular weight, chemical composition, crystallinity, surface area) and also by the inherent characteristics of a specific enzyme (activity, stability, local concentration, amino acid composition, and 3-D conformation). It is also very important to take into account the medium conditions such as pH and temperature, since they influence both the properties of the substrate and of the enzyme. The presence of stabilizers, activators, or inhibitory products in the medium, resulting from material degradation or leaching out of processing additives, may affect the enzyme-catalyzed reactions by influencing enzyme adsorption and activity.

The enzymatic hydrolysis of solid substrates is normally characterized by an enzyme saturation point; at this enzyme concentration, no further increase in the degradation rate is observed when more enzyme is added. This has been attributed to a decrease in the exposed polymer surface as the enzyme molecules saturate the surface and appears to be limiting the progress of degradation of some biomaterials. Such behavior was not observed by Tang et al.,[26] who studied the effect of enzyme concentration (cholesterol esterase, CE) on the degradation rate of polycarbonate polyurethanes (PCNUs). These authors found that the degradation of PCNUs was highly dependent on enzyme dose, and the dose response was influenced by the surface chemistry and structure of the polymer.

The chemical modification of polymers (crosslinking, removal, or introduction of chemical groups in the polymer chain) also affects the enzymatic degradation rates since, depending on the degree of chemical modification, it may compromise the ability of the enzyme to recognize the modified substrate. This seems to be the case of lysozyme (enzyme responsible for the degradation of peptidoglycan and also chitin materials), which exhibited low activity toward chitosans with high degrees of deacetylation[27,28] or crosslinked chitosan.[29]

It was shown that different hard segment distributions at the surfaces of polyether-urea-urethanes could influence the manner in which the enzyme cholesterol esterase adsorbs, binds, and expresses its activity on the surface of the polymers.[30–32] It was found, for instance, that as hard segment increased, the enzyme hydrolytic activity was reduced.[32]

The complex chemical nature of body fluids and inflamed tissues around the implant, the variability in the polymer material, and the variability associated with biological systems (e.g., rate of metabolism of the host) make the overall degradation of biomaterials a rather complicated process. The first interaction of an implanted biomaterial with the host tissue is the adsorption of proteins. The subsequent interactions are determined by the nature of the proteins adsorbed. Blood plasma contains over 150 proteins, and any of these may adsorb to the biomaterial, depending on the binding potential of the particular protein.[33] Proteins may adsorb to a biomaterial surface with low affinity and may be replaced by other proteins with higher binding affinities. The deposition of proteins on the surface of biomaterials is followed by adherence of certain type of cells.

The adsorption of other proteins to the polymer surface may influence the enzymatic degradation of biomaterials. It was shown, for example, that the preadsorption of fibrinogen (Fg) onto the modified and nonmodified surfaces of poly(ether-urethane) (PEU) polymers provided a temporary protective effect against the hydrolytic action of cholesterol esterase (CE) enzyme.[34] This effect may be explained by the fact that the prior adsorption of Fg on the polymer surface might have occupied sites for CE adsorption, consequently limiting the access of the enzyme to the susceptible hydrolysable bonds. Another explanation may be related to the inability of the degradation products to release from the polymer surface due to the adsorbed layer of Fg in the immediate area.

12.3.3.2 Potential Enzymes Involved in the Degradation of Biodegradable Polymers, Their Activities, and Half-Lives in Human Plasma

The enzymes present in serum can be divided into two categories[35]: (1) plasma-specific enzymes and (2) non-plasma-specific enzymes. The former are enzymes whose normal function is related to blood coagulation, complement activation, and lipoprotein metabolism. The latter are enzymes that have no physiological function in the plasma, whose cofactors or even substrates are not normally present in the plasma. This category includes enzymes that are secreted by tissues — e.g., amylase, lipase, phosphatases — and also enzymes associated with cellular metabolism.[35] Their presence in the serum at low levels may be due to the turnover of cells within the tissue causing release of the enzyme. The determination of enzyme activities in plasma has been used for clinical diagnosis since high levels of certain enzyme activities in serum may indicate tissue damage or malfunction. It should be noted, however, that increases in enzyme activities might also be due to other factors such as increased cell turnover, cellular proliferation, or decreased clearance by the kidney.[35]

Many of the enzymes released into the serum are normally removed at a fairly rapid rate having low half-lives.[35] Table 12.1 gives some reference values for the activities of some enzymes as well their half-lives in serum.

The initial stages of certain diseases and the presence of internal injuries give rise to elevated levels of enzyme concentrations in body fluids (lymph, blood, and urine).[35] Inflammation usually occurs at the biomaterial–tissue interface and reflects surface adsorption of plasma proteins, complement activation, neutrophil and macrophage infiltration, hyperplasia, and release of inflammatory mediators, free radicals, and proteolytic enzymes.[33] The major enzymes associated with inflammatory cells are indicated in Table 12.2. The concentration and composition of enzymes around the implant depends on the progress of the inflammatory reaction, since each cell synthesizes specific lysosomal enzymes.

An intrinsic characteristic of natural and natural-origin polymers is their ability to be degraded by naturally occurring enzymes, which may indicate the greater propensity of these materials to be metabolized by the physiological mechanisms.[17]

TABLE 12.1
Reference Concentration and Half-Lives of Some Enzymes in Human Serum

Enzyme	Concentration	Ref.	Half-Life (h)	Ref.
Lysozyme	4–13 mg/l	27	16[a]	39
α-Amylase	46–244 U/l	36	9.3–17.7	40
Lipase	30–190 U/l	37	6.9–13.7	40
Alkaline phosphatase (bone)	11 mg/l	38	30–50	35

[a] After injection into HeLa cells.

TABLE 12.2
Potential Cellular Enzymes Involved in Inflammatory Response

Enzyme	Activity/Function in Wound Healing
1. Proteolytic enzymes	Hydrolysis of proteins, acting as an aminopeptidase (cleaving peptides bonds at specific amino acid residues).
1.1 Cathepsins (B, G, H) (EC 3.4.22.1)	1.1 Cathepsin B catalyses the hydrolysis of proteins with broad specificity for peptide bonds. Thiol protease is believed to participate in intracellular degradation and turnover of proteins.
1.2 Elastase (EC 3.4.21.11)	1.2 Hydrolysis of proteins, including elastin, cleaving preferentially at Val-|-Xaa > Ala-|-Xaa. Medullasin modifies the functions of natural killer cells, monocytes, and granulocytes.
1.3 Neutrophil Collagenase (EC 3.4.24.34)	1.3 Cleavage of interstitial collagens in the triple helical domain. Can degrade fibrillar type I, II, and III collagens.
1.4 Trypsin (EC3.4.21.4)	1.4 Cleavage of peptide bonds preferentially at Arg-|-Xaa, Lys-|-Xaa. Reduces inflammation by dissolving blood clots and extracellular protein precipitates.
2. Hydrolysis of glycosides	
2.1 Lysozyme (EC 3.2.1.17)	2.1 Hydrolysis of 1,4-β-linkages between *N*-acetyl-*D*-glucosamine and *N*-acetylmuramic acid in peptidoglycan of the prokaryote cell walls. Lysozymes have primarily a bacteriolytic function; those in tissues and body fluids are associated with the monocyte-macrophage system and enhance the activity of immunoagents.
2.2 β-*N*-acetyl-*D*-hexosaminidase (EC 3.2.1.52)	2.2 Hydrolysis of nonreducing end *N*-acetyl-*D*-hexosamine residues in *N*-acetyl-β-*D*-hexosaminides. It is responsible for the degradation of GM2 gangliosides and a variety of other molecules containing terminal *N*-acetyl hexosamines, in the brain and other tissues.
2.3 β-*D*-glucuronidase (EC 3.2.1.31)	2.3 Hydrolysis of glucuronic acid esters. Found diffusely in macrophages and fibroblasts, and in granular form in eosinophilic granulocytes at the implantation site.
3. Hydrolysis of lipids	
3.1 Lipase (EC 3.1.1.3)	3.1 Hydrolysis of triglycerides to partial glycerides and fatty acids. Present in macrophage cells.
3.2 Phospholipase A2 (EC 3.1.1.4)	3.2 Catalyzes the release of fatty acids from phospholipids. It has been implicated in normal phospholipid remodeling, nitric oxide-induced. Together with its lysophospholipid activity, it is implicated in the initiation of the inflammatory response.
3.3 Cholesterol esterase (EC 3.1.1.13)	3.3 Enzyme with broad specificity, acting on esters of sterols and long-chain fatty acids. Found in monocyte-derived macrophages (MDM). It is released from the lysosomes when MDM are activated during chronic inflammatory response.
4. Others	
4.1 Acid phosphatase (EC 3.1.3.2)	4.1 Hydrolysis of phosphate esters. The acid phosphatase in normal serum is derived from blood platelets or lysis of erythrocytes and is indicative of macrophage activity at the implant site.
4.2 Alkaline phosphatase (EC 3.1.3.1)	4.2 Hydrolysis of organic phosphate esters. Enzyme associated with plasma membrane.

Data from Price, N.C. and Stevens, L., *Fundamentals of Enzymology. The Cell and Molecular Biology of Catalytic Proteins*, 3rd ed., Oxford University Press, Oxford, 1999, chap. 8, 10; Cassim, B., Mody, G., and Bhoola, K.D., *Pharmacol. Therap.*, 94, 1, 2002; Duguay, D.G. et al., *Polym. Degrad. Stabil.*, 47, 229, 1995; Labow, R.S., Erfle, D.J., and Santerre, J.P., *Biomaterials*, 16, 51, 1995; Erfle, D.J., Santerre, J.P., and Labow, R.S., *Cardiovasc. Pathol.*, 6, 333, 1997.

For instance, the degradation of hyaluronic acid in mammals is carried out by the concerted action of three enzymes[45,46]: hyaluronidase, β-*D*-glucuronidase, and β-*N*-acetyl-*D*-hexosaminidase.

Starch and its derivatives have been widely used in the pharmaceutical industry as controlled-release excipients for the preparation of matrix tablets,[25,48] since starch can be enzymatically

degraded by α-amylase and other amylolytic enzymes to form water-soluble oligosaccharides, resulting in polymer erosion with the concomitant drug release.[25,48] It was also shown that α-amylase was even able to degrade chemically modified starch.[48] A more detailed description about the enzymatic degradation of starch-based biomaterials will be discussed in Section 12.5.

The main enzyme involved in the degradation of chitin derivatives in the body is lysozyme.[27] Although the natural substrate of lysozyme is peptidoglycan, a negatively charged polysaccharide present in the bacteria cell walls, it has been shown to have the ability to cleave glycosidic linkages of chitin derivatives to some extent, but its activity strongly depends on the degree of chemical modification.[27–29]

Labow and coworkers[22] had studied extensively and characterized the enzymatic hydrolysis of polyether-polyurethanes (PEU), polyester-polyurethanes (PESU), and polycarbonate-polyurethanes (PCNU) by cholesterol esterase (CE), carboxyl esterase, elastase, proteinase K, thrombin, cathepsin B, and phospholipase A_2 enzymes. Although enzymes like elastase have been shown to be involved in the biodegradation of PUs, CE was the enzyme showing the highest activity toward these polymers,[22] and further studies were carried out with this enzyme to study the effect of several parameters on the enzymatic behavior of PUs.[26,30–32,34,42–44,49–61]

The enzymatic hydrolysis of poly(*L*-lactic acid) (PLLA) polymers has been studied using proteinase K,[62–64] an endopeptidase enzyme responsible for the hydrolysis of peptides amides in keratin and other proteins. It was demonstrated that this enzyme was able to accelerate the hydrolysis of PLLA.[62] PLLA and poly(glycolic acid) (PGA) are polymers synthesized from materials found naturally in the body, and therefore they are expected to be degraded by enzymes. Schakenraad et al.[65] observed a slightly increased level of lactate dehydrogenase (LDH, an enzyme involved in the conversion of lactic acid) and its coenzyme NADH-reductase in macrophages and fibroblasts after 7 days of PLA implantation in AO/BN rats. It was suggested that the increase in LDH activity might be related to the release of *L*-lactic acid monomers and oligomers as a result of PLA degradation. This means that degradation of biomaterials may induce other enzyme activities besides the enzyme patterns observed in the early stages of implantation, which are simply related to wound healing reaction.

Lipase is an enzyme that is able to catalyze the hydrolysis of ester bonds in polyesters when in the presence of an aqueous media.[63] It was found that certain lipases enhanced the degradation of polycaprolactone (PCL) when compared with incubation in buffer only.[66,67] The enzymatic hydrolysis of PCL occurs mainly at the polymer surface since it is difficult for a hydrophilic enzyme to diffuse into a hydrophobic polymer like PCL.[37] The surface erosion mechanism of PCL demonstrated by lipase enzyme may be useful to deliver bioactive agents by an enzymatically controlled process.

Polyphosphates are a class of biodegradable polymers with a phosphodiester backbone, which is prone to hydrolytic enzymes such as phospholipases and phosphodiesterases.[68] Renier and Khon[68] studied the degradation kinetics of a biodegradable polyphosphate in fetal bovine serum (FBS) and postulated that alkaline phosphatase present in serum may cleave the phosphodiester linkage of the backbone of polyphosphate polymers.

12.4 *IN VITRO* STUDIES TO ASSESS THE DEGRADATION KINETICS OF BIODEGRADABLE POLYMERS

In vitro degradation tests of biodegradable polymers in simple aging media are normally conducted to predict the performance of such polymers in the clinical situation. Taking into account the complexity of the body fluids, it is common to find different results when the same materials are studied both *in vitro* and *in vivo*. For instance, it has been observed that *in vivo* degradation rates of a linear copolymer of lactide and ethylphosphate[69] and of poly(lactide-co-glycolide) microspheres[11] were faster *in vivo* than *in vitro*. The higher *in vivo* degradation rates of biomaterials

have been explained by the effects caused by cellular and enzymatic activities found in the body and to the dynamic loads experienced by devices during the implantation period. Opposite behaviors were found by Jiang and coworkers[70] who reported that the *in vivo* degradation of poly(ester-anhydrides) based on aliphatic and aromatic diacids was slower than *in vitro*. The lower *in vivo* degradation might be related to the prevention of water penetration and diffusion of degradation products by the formed capsule around the implanted materials *in vivo*. Hooper et al.[71] found, however, similar *in vitro* and *in vivo* degradation rates of tyrosine-derived polymers (poly[DTE carbonate] and poly[DTE adipate]), which confirmed the absence of enzymatic hydrolysis in the degradation process of these materials. The differences found between *in vitro* and *in vivo* results are possibly related to the intrinsic properties of each biomaterial and with the *in vitro* systems used to simulate the *in vivo* conditions. Nevertheless, *in vitro* results may constitute a useful approach to predict the degradation rate of biomaterials and also a guideline for planning *in vivo* studies.

Most of the degradation studies reported in the literature are performed by incubating the material in phosphate buffer saline (PBS) solution. In this way, it is expected that only degradation by normal hydrolysis will occur. Hooper and colleagues[71] found that both PBS and simulated body fluid (SBF) solutions were appropriate incubation buffers for an accurate simulation of the *in vivo* degradation of tyrosine-derived polymers. Nonbuffered solutions (water, isotonic saline solutions) have been also used in many degradation studies, but these tests had ignored the buffer capacity of body fluids. Furthermore, many of the degradation tests do not contemplate the renewal of the degradation medium, and again, such testing protocols do not allow for the evacuation of degradation products by fluid flow, intrinsic to *in vivo* conditions.[9]

Another important issue is the mass/volume ratio used in the degradation tests. Different mass/volume ratios have been reported in the literature, and this may affect the degradation kinetics of biomaterials. It is known, for instance, that articular cartilage tissue is quite avascular,[9,72] which means a low level of surrounding fluids.

The choice of the incubation media, mass/volume ratio, and duration of the *in vitro* degradation studies should take into account the locale where the materials will be implanted and the time that the materials will be in contact with the tissues and body fluids. For instance, the release of acidic degradation by-products in anatomical regions, without access to sufficient quantities of body fluids, might overwhelm the capacity of the body fluids to ensure local buffering and cause adverse effects to the surrounding tissues.[9] Nevertheless, the use of other degradation solutions (water, serum, enzyme buffer, ionic and simulated body fluid solutions) may provide insight into some aspects of the degradation process of certain biomaterials since enzymes and other reactive species are expected to be present in the *in vivo* environment (please see Section 12.3.3.2).

Another focus of controversy is the use of static or agitation conditions during the degradation tests. The level of agitation may influence the degradation kinetics of biomaterials since it facilitates the release of degradation products from the bulk or surface of the material to the solution. Furthermore, in the case of enzyme-mediated degradation, agitation promotes the contact between the soluble free enzyme and the insoluble substrate (biomaterial) enhancing adsorption. It is assumed that the body fluids move slowly in soft and hard tissues, and it is suggested that degradation tests should be performed under static[16] or slow agitation (two rotations per minute)[73] to mimic the physiological conditions. On the other hand, the use of different agitation conditions during the *ex vivo* growth of tissues (static flasks, mixed flasks, rotating vessels, flow-perfusion bioreactors) has been reported. It was observed that cartilage constructs cultured in a dynamic laminar flow field in rotating vessels led to better results (in terms of size, mechanical properties, and higher content of collagen and glycosaminoglycan) than static and agitated flasks.[74] In addition, it is expected that biomaterials will experience some fluid flow and repetitive loading when under *in vivo* conditions,[74] and this is likely to influence the degradation kinetics of biodegradable polymers. The study of the degradation behavior of biomaterials under these different incubation conditions is, therefore, important to investigate the adequacy and efficacy of the material during

cell culture and implantation. Furthermore, these studies may also provide additional information about the degradation mechanisms of the materials.

Agrawal et al.[74] studied the effect of fluid flow on the *in vitro* degradation kinetics of poly(*D,L*-lactide-co-glycolide) (PLG) scaffolds. Contrary to what might be expected, it was observed that the fluid flow decreased the degradation rate significantly. This result was attributed to the absence of the autocatalytic effect[6,10,16,63] caused by the acidic by-products formed during hydrolysis of PLG copolymers under flow conditions, since in this situation it is likely that the degradation products are washed away. Different results may be obtained when studying other polymeric systems, since it is expected that the effect of fluid flow may cause additional mechanical stress and contribute to the leaching out of processing additives and fast release of degradation products, leading to increased degradation. The degradation rate of a particular biomaterial under these conditions may not, however, follow any of these behaviors, and only an accurate test may give indications on the type of degradation.

The number of samples tested should always allow for a valid statistical analysis ($n > 10$), and a proper control for each test period should always be used.

The great diversity of the conditions used in the various degradation studies does not allow for direct comparisons to be made, and the use of standardized methods for characterizing the degradation properties of biomaterials would be advisable. The ISO standards 10993-9[75] and 10993-13[76] provide guidance on general requirements for the design of tests to assess the degradation of biomaterials and to identify and quantify the degradation products from polymers, respectively. It is also mentioned in these standards that, due to the wide range of polymeric materials used in biomedical applications, the degradation tests should be carried out under conditions that closely simulate the environment in which the material is going to be used and that conditions should reflect the intended function of the material.

12.4.1 Degradation-Monitoring Techniques

After implantation, biodegradable polymers may undergo a variety of changes in their physicochemical properties as a result of degradation. These alterations may compromise their desired function and evoke undesirable tissue response. It is important, therefore, to characterize and quantify the changes that occur in the biomaterial at different stages of degradation and to evaluate the corresponding tissue response, since the tissue reaction may change during the course of the degradation process. Hooper et al.[71] observed that the tissue response of poly(*L*-lactic acid) and poly(DTE adipate) after 60 days was not significantly different but, for the same degree of degradation, evident differences on the tissue response were found.

In the early stages of degradation, the event with most significance is the gradual diffusion of water solution into the polymer matrix (leading to an increase of water uptake), while little changes occur in the sample mass, in the molecular weight, and consequently in the polymer tensile strength. The main changes are related to the ones occurring at the polymer surface, such as an increase of the surface roughening and surface free energy and eventually some chemical changes due to hydrolysis. The second stage of the degradation process is characterized by a decrease in the polymer molecular weight, as a result of chain scission and by the diffusion of larger-molecular-weight fragments to the solution. This leads to increased weight loss and porosity of the matrix and loss of tensile strength. Advanced stages of degradation are characterized by collapse of the polymer matrix and dramatic decrease in molecular weight and an increase in weight loss. Crystallinity substantially disappears, and as a consequence, there is a marked decline in the mechanical properties.[77]

The main techniques used to evaluate the degradation of biomaterials can be divided into surface analysis (infrared spectroscopy, X-ray photoelectron spectroscopy, contact angle measurements), which are more appropriate to monitor the changes occurring in the first stages of degradation, and bulk analysis (determination of changes in molecular weight, weight loss, temperature transitions,

mechanical properties) for characterizing the later stages of degradation. Therefore, the selection of the characterization techniques should be made based on the degradation stage and also on the unique properties of the biomaterials under investigation. Some of the most common properties and techniques used to evaluate the course of degradation of biodegradable polymers are described in the following section.

12.4.1.1 Water Absorption and Weight Loss

The main factors influencing the wettability of polymers are related to their degree of crystallinity, chemical composition, and aqueous media. Marois et al.[78] found different water absorption behaviors for polyhydroxyoctanoate (PHO) films in water and in PBS solution. It was observed that incubation in PBS led to lower water absorption than when incubated in water, and this difference was attributed to osmotic phenomena and to the ionic strength of the PBS. The same behavior was obtained by Li[16] with PLA_{50} (L-LA/D-LA, 50/50) polymers.

Water uptake measurements can give, therefore, some indication of the hydrophilicity/hydrophobicity nature of the materials and therefore of their tendency to be degraded by hydrolysis. The water uptake values are normally obtained after equilibrium of the materials in solution. In some cases, the equilibrium cannot be reached because the material is degrading at the same time. In this case, the level of water uptake usually increases with the degradation time due to an increase in the permeability of the materials (a porous structure is normally obtained as a result of the release of degradation products).

During degradation, the mass of the material may undergo changes, and these changes can be monitored by comparing the mass before and after the degradation period. The material should be dried to a constant mass before measuring the initial weight of the sample to avoid residual moisture in the samples. The drying temperature should not exceed the temperature at which the materials undergo irreversible changes (e.g., melting temperature). After degradation, the sample should be washed thoroughly with distilled or deionized water to remove traces of soluble degradation products, enzymes, salts, or other impurities and dried under vacuum conditions until constant weight. The extent of degradation is commonly determined by calculating the percentage of weight loss.

12.4.1.2 Molecular Weight

The determination of the polymer molecular weight (M_w) during the degradation process is one of the most important analyses to be made when studying the degradation mechanisms of polymeric biomaterials. Many other properties are affected by changes to the M_w, including mechanical properties, crystallinity, weight loss, and morphology. The evolution of M_w during degradation can be determined by gel permeation chromatography (GPC) or by viscometry techniques. The main useful parameters to be analyzed are the number-average (M_n) and the weight-average (M_w) molecular weights. The ratio M_w to M_n is known as the polydispersity index, which measures the breadth of the molecular weight distribution.

12.4.1.3 Crystallinity

In semicrystalline polymers, an increase in polymer crystallinity has been frequently observed at early stages of degradation,[8,16,79] and this is likely related to the plasticizing effect of the absorbed water that makes chain movements possible, allowing crystallization to proceed toward thermodynamic equilibrium.[78] On the other hand, initial hydrolysis takes place at the amorphous regions, since these regions are more accessible to water molecules and enzymes, leading to an initial increase in the crystallinity of the polymer.[15,16] The generation of crystallized monomers and oligomers was also indicated as a source for increased crystallinity after degradation.[8] As chain

scission occurs in the crystalline regions, during later stages of degradation, a decrease in the overall crystallinity of the polymer can be expected.[77]

The changes in the crystallinity of biodegradable polymers after degradation can be followed, among other techniques, by differential scanning calorimetry (DSC) or by wide-angle x-ray diffraction (WAXD).

The analysis of crystallinity conducted by DSC is made based on changes in the glass transition temperature (T_g), crystallization temperature (T_c), and melting temperature (T_m) and on the heat of fusion (ΔH) values.

The shift, disappearance, or appearance of new and broader peaks in WAXD patterns is an indicator of a change in the crystallinity.

The percentage of crystallinity may be derived from DSC and WAXD data using empirical correlations.

12.4.1.4 Morphology and Dimensional Changes

Changes in the surface morphology of biomaterials after degradation, like roughness and the appearance of cracks or micro/macropores, may be examined by microscopy methods such as light microscopy, scanning electron microscopy (SEM), and atomic force microscopy (AFM). In addition, AFM gives information about the sample topography at low magnifications and allows for roughness calculations.[59]

The measurement of sample dimensions during the degradation process may be an important parameter to be analyzed since, in some cases, degradation can cause dramatic morphologic changes that may compromise the macroscopic properties of the final implant (e.g., bone cements or other filler/fixation devices). This analysis can also provide information about the mode and direction of the degradation font.[80] Changes within the material may be analyzed by observing the sample cross section obtained by freeze-fracturing in liquid nitrogen.

12.4.1.5 Surface Chemistry

The surface chemistry of biodegradable polymers, before and after degradation, can be analyzed by several techniques that include Fourier transform infrared (FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS), and contact angle measurements. The main difference between these techniques is related to the amount of information provided and the depth of sample analyzed. XPS analysis can give information about the elemental and chemical group composition at the material surface (first 10 nm of surface), and the depth of penetration can be varied by the takeoff angle, allowing the construction of depth profiles over the outermost polymeric surface.[51,58] FTIR with attenuated total reflectance (ATR) can provide information for chemical groups within the top 5 μm of the surface.[3,30] In addition, reflection mode (FTIR-ATR) allows for the analysis of samples in the wet state, which is more relevant from the biological point of view.[30,31] The ability to characterize the chemical state of wet surfaces is rather useful to analyze the mechanism by which water could penetrate and disturb the structure of the materials. It is also known that polymer surfaces gradually change toward a more hydrophilic structure in polar aqueous environments.[81] For example, it was observed by Tang and colleagues[31] that, after exposure to water, some polycarbonate-polyurethanes showed an increase in hydrogen-bonded carbonate carbonyls.

Contact angle measurements may give an indication on changes in the hydrophilicity of the material surface. Higher contact angle values (which can be obtained in static sessile drop or dynamic conditions using both the advancing and the receding contact angle) indicate generation of a more hydrophobic surface.

The choice of technique depends, therefore, on the amount of information provided by each technique, its availability, and its associated cost.

Surface chemistry characterizing techniques may give indications about the type of degradation mechanism.[7,70,78,82] Marois et al.[78] have used FTIR to analyze chemical changes in the structure of polyhydroxyoctanoate (PHO) films after degradation in aqueous media, and this technique indicated that the possible degradation mechanism of this material began internally, moving outwardly to the surface.

12.4.1.6 Mechanical Properties

Certain biomedical applications, such as hard tissue replacement, require biodegradable materials with mechanical properties typically on the same order of magnitude as those of the tissue that it replaces. In addition, the material should degrade while maintaining a specified minimum mechanical strength to support the formation of new tissue. Thus, in these cases, it is critical to evaluate the mechanical performance of biomaterials during the course of their degradation. Currently, the techniques described in the American Society for Testing and Materials (ASTM) standards have been the most commonly used for testing tensile, bending, and compressive properties of polymeric biomaterials.[83]

12.4.1.7 Isolation and Identification of Degradation Products

The development of materials for biomedical applications always requires extensive biological testing to demonstrate the safety of both the material and its degradation components.[84] A potential disadvantage of using biodegradable polymers in biomedical applications is the eventual toxicity of the degradation products. If the biomaterial degrades, either spontaneously or due to biological activity, components can leach into surrounding tissues and may enter the circulation and be easily metabolized via normal pathways, causing toxic effects systemically and in distant sites.[33] It is necessary, therefore, to identify the major species produced at different stages of degradation and the kinetics of their formation. In addition, the release and accumulation of degradation products may interfere with the course of degradation and with the equilibrium of degradation reactions. The release and accumulation of some degradation products may accelerate the degradation rate of some biomaterials. For example, the release of carboxyl end groups formed by chain cleavage of lactide and glycolide aliphatic polyesters during hydrolysis may produce a decrease in the local pH and further catalyze the hydrolysis of other ester bonds, a phenomenon called autocatalysis.[6,10,16,63,80,83] On the contrary, Yaszemski et al.[85] observed that the mechanical properties of a poly(propylene fumarate) (PPF)-based composite material increased with degradation time, which was explained by a crosslinking effect promoted by complexation between carboxylic groups, formed from PPF degradation and accumulated in the incubation solution, with divalent calcium ions released from β-tricalcium phosphate (β-TCP). Another possibility is the potential inhibitory effect of degradation products on enzyme-catalyzed reactions. For example, it is known that the hydrolysis by-products of starch (maltose and maltotriose) strongly inhibit the activity of α-amylase.[25] The release of processing additives or other degradation products may also have a denaturation effect on enzyme activity. Taking into account the occurrence of such effects, the renewal of degradation liquors may have, therefore, a significant impact on the degradation kinetics of certain biomaterials.

The content of degradation solutions depends mainly on the material tested, the degradation stage, and the incubation solution used in the degradation studies. It might contain degradation products, enzyme, proteins, enzyme impurities, salts, and debris. The degradation solutions can be subjected to appropriate chemical and physical analysis such as high-performance liquid chromatography (HPLC), UV-visible spectrophotometry, nuclear magnetic resonance (NMR), mass spectroscopy (MS), or other analytical methods to identify and, if possible, quantify the main degradation products. It may be necessary, before these analyses, to perform separation techniques for the removal of salts, processing additives, monomers, oligomers, residual solvent released into the

degradation solutions, or proteins and enzymes, since the presence of these components may interfere with the identification and quantification of degradation products. Ultrafiltration membranes can be used to separate high-molecular-weight components, such as proteins, from water-soluble low-molecular-weight degradation products in the incubation media.

Analysis and quantification of the degradation products released into the incubation medium also provide information about the modes of action of enzymes on the polymer and what linkages are more susceptible to hydrolysis. The enzymatic degradation of polyurethanes has been assessed by means of following the radioactive release of ^{14}C-labeled degradation products.[26,30–32,34,42–44,49–61] The determination of soluble reducing sugars may be used to assess the enzymatic hydrolysis of polysaccharides.[48,86]

HPLC and MS techniques have been successfully used on the isolation and identification of the degradation products of biodegradation of polyurethanes by cholesterol esterase.[50,56,57]

Chaubal and coworkers[69] followed the generation of water-soluble degradation products during the *in vitro* degradation of a linear copolymer of lactide and ethylphosphate by NMR.

12.4.2 Mechanisms of Degradation

In general, the first degradation event after contact with water molecules is the hydrolytic scission of the polymer chains leading to a decrease in the molecular weight. At this initial stage, the first degradation products are not small enough to become soluble, and no significant change in the material weight is detected. With increasing time, the molecular weight of degradation products is reduced by further hydrolysis, which allows them to diffuse from the bulk material to the surface and then to the solution, causing significant weight loss.

Polymers can be degraded by bulk degradation[6,8,9,62] or surface erosion mechanisms.[6,79,80,82,87] Bulk degradation is characterized by hydrolysis of chemical bonds in the polymer chain at the center of the material, which typically results in an empty shell but maintains their size for a considerable portion of time.[16] This degradation behavior has been observed for polylactides polymeric systems, whose degradation mechanisms are well described in the literature.[6,8,15,16]

Degradation by surface erosion mechanism is characterized by loss of the material from the surface only, resulting in very predictable mass loss profiles. The materials get smaller but keep their original geometric shape. This feature may be beneficial for delivering molecules at constant rate and maintaining the mechanical and structural integrity of the material with degradation. Poly(anhydrides) and poly(orthoester)s are examples of surface-eroding polymers.[6,79,80,87]

Enzyme-catalyzed degradation of polymeric biomaterials may follow a surface erosion mechanism, especially for highly crystalline and hydrophobic homopolymers. Due to their relatively large size, the enzyme molecules cannot penetrate the tightly packed structure of certain polymers, so that the enzymatic catalysis occurs at the polymer–enzyme interface. As the degrading surface becomes roughened or fragmented, enzymatic action may be enhanced as a result of increased surface area. Enzymatic degradation mechanisms of polymeric materials depend, however, on many other factors such as chemical composition, degree of homogeneity, and processing technique.

Several different types of models have been applied to describe the degradation kinetics of biodegradable polymers,[2,6,10,42,68,88] assuming first- or second-order kinetics. Recently, computer modeling has also been used, but some of these models did not consider diffusion theory to describe transport phenomena.[6] The mathematical model proposed by Duguay and coworkers[42] describes, in a very complete and comprehensive way, the *in vitro* enzymatic degradation of biomedical polyurethanes by a single enzyme.

Despite the progress made on mathematical models to predict the degradation of biodegradable polymers, much more data and sophisticated models are needed to apply these approaches to other polymeric systems.

12.4.3 Strategies for Controlling the Degradation Rate of Biodegradable Polymers

Depending on the application (long-term implants or temporary matrices for tissue engineering and drug delivery), three main strategies have been observed to alter the degradation rate of biodegradable polymers. Some strategies intend to increase the degradation rate of biomaterials, and this is normally obtained by blending or copolymerization techniques or by adjusting the polymer physicochemical properties. The reduction of the degradation is mainly achieved by selecting the polymer chemical composition (high degree of crystallinity, hydrophobicity, elevated molecular weight, nonhydrolysable bonds) and by using surface modification methods. The third approach may be obtained by selecting the right combination of the other two strategies and may take advantage of using smart systems, which means that polymers will degrade only in response to a certain stimulus.

The degradation of some biomaterials involves nonenzymatic hydrolysis, which is largely nonregulated.[89] In such situations, only the calendar may determine the rate of resorption of the material, and in these cases, the degradation of biomaterials is controlled by manipulating their physicochemical properties. Blending and copolymerization have been used to change the degradation rate of polymeric materials, since, in general, blends and copolymers degrade faster than homopolymers from the same family.

Other approaches[90,91] have been used, such as including enzymatic recognition sites in polymer backbones (e.g., amino acids) to enzymatically modulate the material degradation or making the material sensitive to the feedback provided by the cells involved in the healing response,[92] as cells enzymatically degrade the extracellular matrix around them.

It has been observed that the inclusion of some substances into polymeric matrices, either as excipients or drugs for controlled delivery, may change the degradation rate of some biomaterials. The inclusion of sodium,[9] calcium,[9] or zinc carbonate[11] into poly(lactide-co-glycolide) (PLG) polymers was shown to retard the degradation of PLG due to the neutralization of carboxylic acid groups formed during PLG hydrolysis by the basic salts, consequently avoiding the autocatalytic effect of the acidic degradation products. Birnbaum and Brannon-Peppas[12] found that the inclusion of increasing amounts of epirubicin HCl in poly(lactic-co-glycolic acid) (PLGA) nanospheres hastened their degradation. This may be caused by an increase in water uptake due to the free space for water to diffuse into the interior of the nanoparticles after drug dissolution and release.

For instance, the combination of a series of surface-modifying macromolecules, containing fluorinated end-groups, into a base polyurethane reduced the material's susceptibility to enzymatic hydrolysis.[51,52]

Kamimura et al.[93] developed a self-regulated degradation system based on dextran (Dex) with a model oxidant dihydronicotinamide (NAH) to achieve nonlinear enzymatic degradation and consequently an oscillative drug release system. The smart system had used the combined activities of three enzymes (dextranase, isomaltase, and glucose oxidase) to generate oxidant degradation products (H_2O_2) able to oxidize NAH-Dex (NA^+-Dex), which was then complexed with carboxymethyl dextran (anionic polysaccharide) to form polyion complexation, consequently eliminating further enzymatic degradation of NAH-Dex.

Materials to be used in some applications, such as hard-tissue replacement, must combine adequate mechanical properties with controlled biodegradability. It may be difficult to achieve the desired combination of degradation and physical properties for a single material. In this context, it may be useful to incorporate specific enzymes into the materials by different immobilization methods to control their degradation rate (this approach will be presented and discussed in the next section).

12.5 ENZYMATIC DEGRADATION OF STARCH-BASED BIOMATERIALS — A CASE IN STUDY

Starch-based polymers have been studied and proposed as potential materials to be used in several biomedical applications.[94–103] They have shown great versatility, are easily processed, and have been proposed for applications such as drug delivery carrier systems,[95–97] hydrogels and partially degradable bone cements,[97,98] materials for bone replacement/fixation or fillers for bone defects,[98] and porous structures to be used as scaffolds in tissue engineering of bone and cartilage.[99] The degradation behavior of starch-based biomaterials in different shapes and compositions (porous structures,[96,99] hydrogels,[95,97] bone cements,[98] chemically modified samples,[100,101] compact samples,[102] composites[103]) has been assessed in different degradation media (namely isotonic saline solution,[96–101,103] with or without bovine serum[102]). The degradation rate of these materials was shown to be dependent on certain parameters such as porosity, hydrophilicity, chemical composition, and degree of chemical modification.[96–101] The susceptibility of these starch polymeric blends to enzymatic degradation was reported recently.[86]

In this section, some experimental work, regarding the susceptibility of starch-based polymers to enzymatic degradation, will be presented and discussed. The main aim of these studies was not to simulate the degradation of the material *in vivo*, but to investigate whether starch polymeric blends could be degraded by certain enzymes with the aim of developing strategies to control their degradation rate by enzymatic means. Furthermore, these studies may also provide insight into the mechanistic aspects of the enzymatic degradation of these materials.

12.5.1 Materials and Methods

The material used in this work was a polymeric blend of corn starch with poly(ε-caprolactone) (SPCL, 30/70 wt%). The blend was processed by conventional injection molding to produce compact discs (Ø = 1 cm). The samples were incubated in phosphate buffer saline (PBS) solution (0.01 *M*, pH 7.4) containing enzyme at concentration of 0.6 mg/ml (lipase from *Thermomyces lanuginosus*, Novozymes A/S, Bagsvaerd, Denmark; α-amylase from *Bacillus amyloliquefaciens*, Genencor International, Rochester, NY, U.S.) at 37ºC with constant shaking at 60 rpm for 6 weeks. A control was also performed by incubating the samples in buffer alone. After degradation, the samples were washed with distilled water and allowed to dry inside a desiccator until constant weight was reached. The surface morphology of the samples after enzymatic degradation was examined using scanning electron microscopy (SEM). The changes in the chemical composition on the surface of starch-based polymers, before and after enzymatic degradation, were analyzed by Fourier transform infrared (FTIR) spectroscopy with attenuated total reflectance (ATR) device.

With the aim of tailoring the degradation rate of starch-based biomaterials, another set of experiments was performed by encapsulating a thermostable α-amylase in the SPCL matrices using a melt-processing method. The encapsulant enzyme was a thermostable α-amylase (Genencor International, Rochester, NY, U.S.) derived from a genetically modified strain of *Bacillus licheniformis*. The lyophilized enzyme was mixed with the polymer in powder form at different weight percentages (0.5 and 5%) and processed by compression molding (P = 4 kg/cm^2, T = 90ºC, 20 min) to prepare capsules (discs of ≈ 0.25 g). A control, without encapsulated enzyme, was also performed. The samples were then immersed in acetate buffer solution (0.1 *M*, pH 5.5) and incubated at 37ºC for different periods of time. Matrix degradation was assessed by determination of weight loss, and the morphology of the sample surface was examined by SEM.

12.5.2 Results and Discussion

Starch is a glucose homopolymer composed of amylose and amylopectin. The former is an essentially linear structure where the glucose units are joined by α(1→4) glycosidic links, while amylopectin consists of linear α(1→4) linked glucose chains, but is a branched molecule with α(1→6)

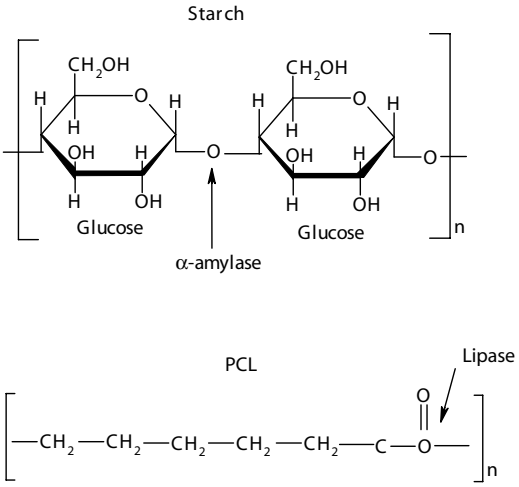

FIGURE 12.1 Schematic representation of the starch degradation by α-amylase and PCL by lipase.

branch points every 24 to 30 glucose residues on average. The main enzymes involved in starch hydrolysis are α-amylases, β-amylases, glucoamylases, α-glucosidases, and other debranching enzymes (pullulanase and isoamylase).[104] α-Amylase is an endo-specific enzyme that catalyzes the hydrolysis of α-1,4-glycosidic linkages of starch to maltose and dextrins, reducing the molecular size of starch (Figure 12.1).[48] In humans, the enzyme occurs in a variety of tissues, but the highest concentrations are in the pancreas and in salivary glands.[35] Low amylase activities are normally detected in the serum (Table 12.1) of healthy subjects, but the concentration of α-amylase in serum increases in individuals with acute pancreatitis or suffering from other disorders (alcoholism, gastrointestinal disease, etc.).[105]

PCL is a biodegradable aliphatic polyester with important applications in the biomedical area whose chemical structure is represented in Figure 12.1. The natural function of lipases is the hydrolysis of triglycerides to partial glycerides and fatty acids. Serum lipase is mainly derived from the pancreatic acinar cells, but other sources of lipase in the human body are the digestive tract, adipose tissue, lung, milk, and leukocytes.[40]

Figure 12.2 shows the IR spectra of SPCL before and after degradation in different incubation solutions. The SPCL spectrum exhibits the characteristic peaks of PCL and starch. The relevant bands of starch are the ones related to OH group (3450 cm^{-1}) and the band at 1150–1040 cm^{-1} corresponding to –C–O–C– of glycosidic bonds.[106] The characteristic peaks of PCL are located at 1740 cm^{-1}, corresponding to the C=O stretch ester carbonyl group. The peaks at 1600–1580 cm^{-1} and 1200–1000 cm^{-1} are related to asymmetric stretch of –COO– and the stretch of –C–O bond at the main polymer chain.[107]

It is possible to observe that, after incubation in PBS solution, no significant changes occurred in the chemical composition of the surface of SPCL material. The effect of α-amylase can be observed by a decrease in the intensity of the peak at 1150–1040 cm^{-1}, indicating the action of α-amylase in cleaving the glycosidic linkages of starch. It is possible to visualize also a decrease of intensity on the peak of OH group. After incubation with lipase, the main effect observed is a significant decrease on the ester band and also on the characteristic bands of the bonds occurring at the polymer chain (1600–1580 and 1200–1000 cm^{-1}). The combination of the two enzymes contributes to significant differences both on the bands of starch and PCL, indicating degradation of both components of the polymeric blend.

The incubation of SPCL in different solutions also causes distinct surface morphologies, which can be seen in the SEM micrographs (Figure 12.3). The incubation in buffer only (control sample) does not cause visible changes in the sample morphology. The sample incubated with α-amylase

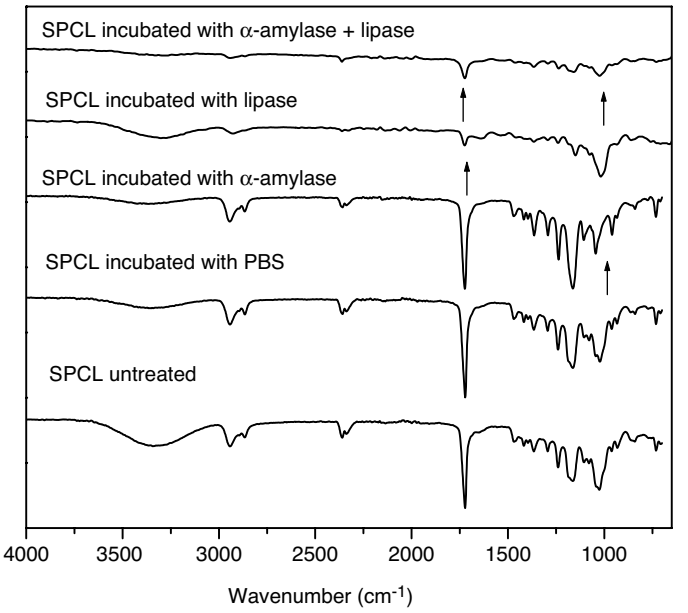

FIGURE 12.2 IR spectra of SPCL material before and after enzymatic degradation with α-amylase and lipase.

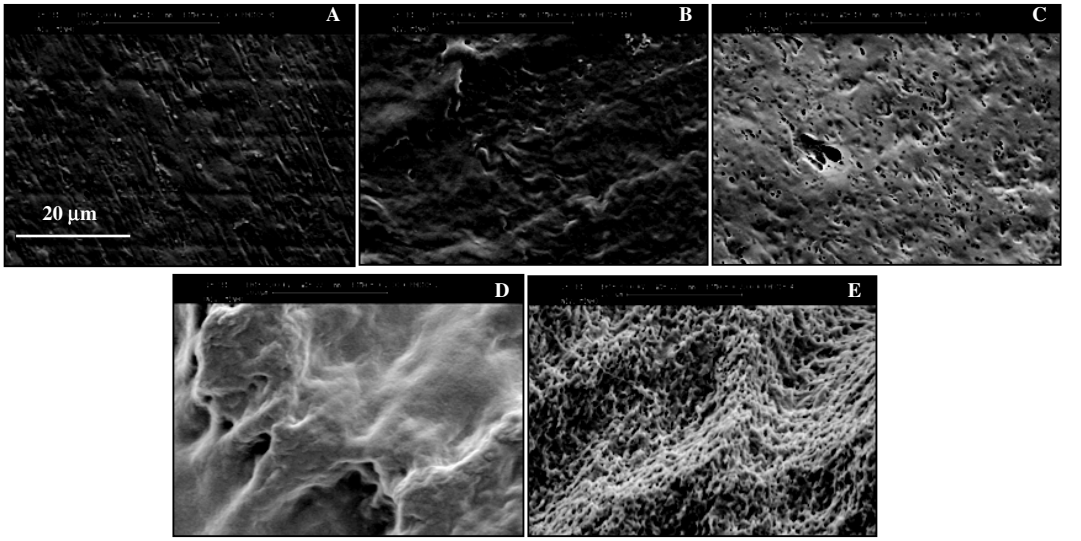

FIGURE 12.3 SEM micrographs of SPCL surface before and after degradation in different incubation solutions for 6 weeks. Magnification ×2000. A) Untreated sample; B) PBS; C) α-amylase; D) lipase; E) α-amylase and lipase.

shows some small pores at the surface, which are probably related to the degradation of starch. Although not visible at higher magnification, samples incubated with lipase exhibited the presence of large fissures on the surface. At high magnification (2000 ×), it is possible to detect changes in the surface topography. Combining both enzymes, it is possible to observe a surface with different topography and with small pores indicating the distinct effects of each enzyme.

Enzyme encapsulation/immobilization can be used to tailor the material degradation and, at the same time, provide controlled-release systems of active organic and inorganic substances at a

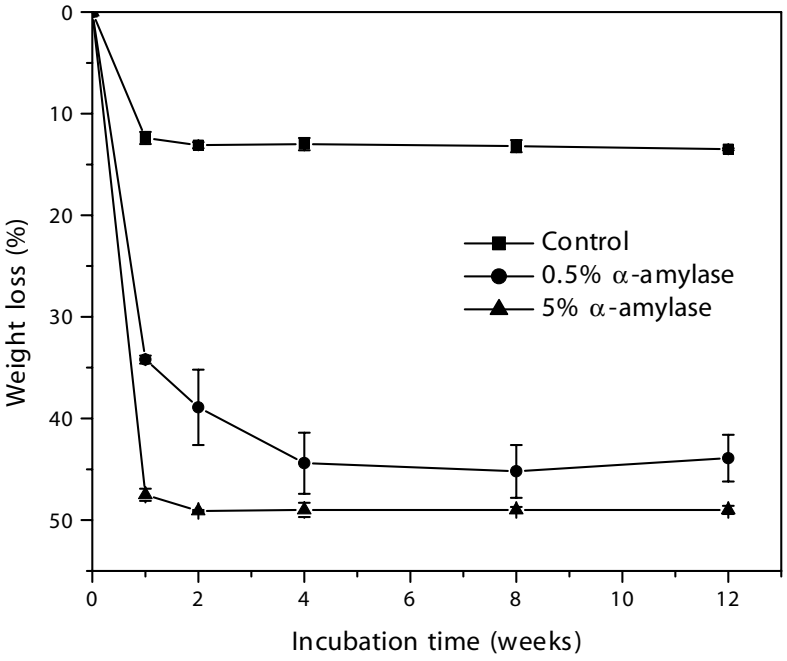

FIGURE 12.4 Degradation profile (pH 5.5, 37ºC) of SPCL discs, measured as weight loss, containing different percentages of encapsulated α-amylase.

desired site and time and at a specific rate. Figure 12.4 presents the degradation behavior of SPCL capsules in the different conditions studied. The control sample exhibits a significant weight loss (about 13%) during the first week, but the sample mass tends to remain almost constant in the following weeks. The original fast weight loss is related to the leaching of plasticizers and to the release of some oligomers due to some thermal degradation of the material that occurs during processing.[86,102,103] When α-amylase was encapsulated, it is possible to observe an increase in the weight loss as a result of the enzyme activity. With 5% of the enzyme encapsulated, almost all starch was hydrolyzed in the first week. These results indicate that the enzyme did not lose its activity during the preparation of the capsules, revealing a nondenaturing effect of the encapsulation technique on the enzyme activity.

The degradation effect of the encapsulated enzyme on the surface of the material can be observed in the SEM micrographs (Figure 12.5), where a highly porous structure is visible. This demonstrates that the enzyme was able to degrade the starch inside the matrix and diffuse from the bulk to the surface to carry out further starch hydrolysis. Furthermore, the encapsulation method seems to be adequate for controlling the degradability of starch-based biomaterials, since the enzyme possesses some degree of mobility, which is important for the degradation of insoluble substrates.

This method can, for instance, be used to incorporate a proper amount of α-amylase in starch-based bone screws, fixation plates, or scaffolds in order to tailor their degradation profile.

Other enzyme immobilization techniques may be used to achieve biomaterials with enzymatically controlled degradation (please see Chapter 17 by Costa et al. in this book). An interesting approach is the incorporation of α-amylase during the formation of calcium phosphate (Ca-P) coatings on starch polymeric blends with the aim of tailoring their degradation rate (please see Chapter 14 by Leonor et al. in this book). It was shown that, using a biomimetic ("nature-inspired") methodology to produce Ca-P coatings on the surface of biodegradable polymers, it is possible to incorporate enzymes without having loss of enzyme activity and at the same time tailor the properties of the coatings (composition, morphology, crystallinity, stability, etc.).

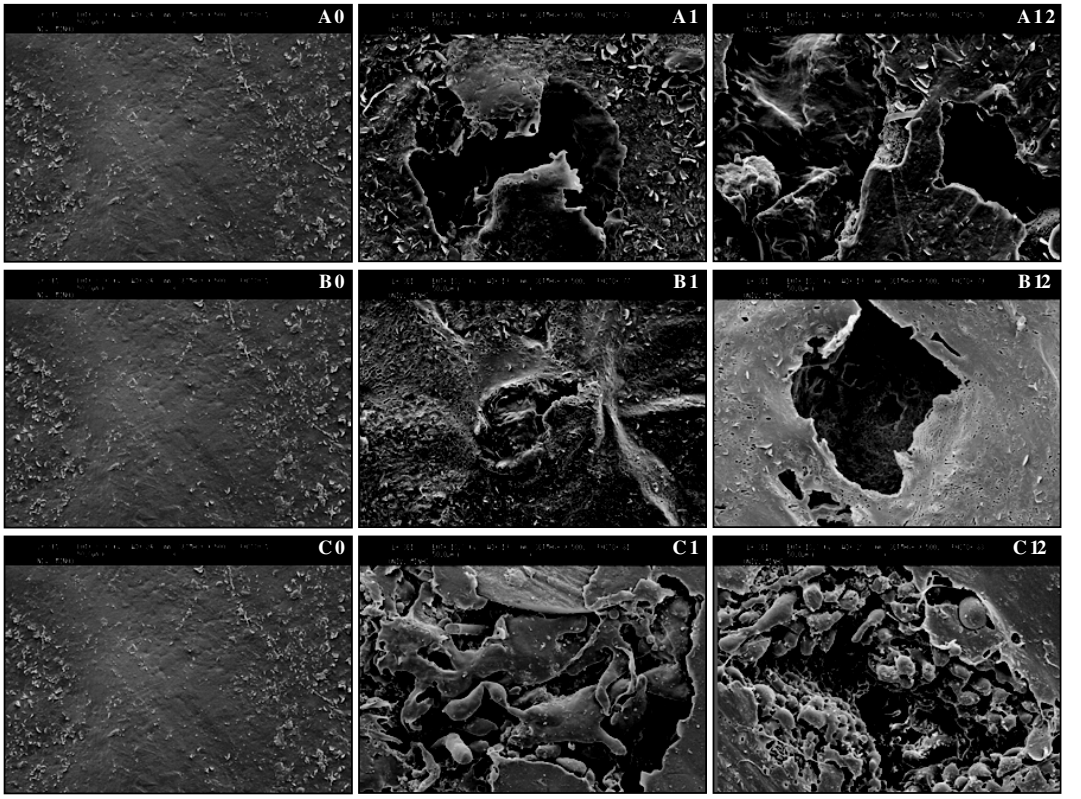

FIGURE 12.5 SEM micrographs of the surface of SPCL discs at different stages (0, 1, and 12 weeks) of degradation (pH 5.5, 37ºC). Magnification ×500. (A) Control sample; (B) sample with α-amylase encapsulated (0.5%); (C) sample with α-amylase encapsulated (5%).

12.6 CONCLUDING REMARKS

Most of the strategies developed to achieve biomaterials with controlled degradation rates have been based on molecular design principles such as the introduction of hydrolysable bonds into polymer backbones, copolymerization and blending techniques, crosslinking, and surface modification methods, depending on the type of application. The development of polymeric systems with a degradation rate controlled by a certain stimulus may constitute, however, a very promising approach with an increasing number of applications in the near future.

For instance, our research group proposed a new strategy to control the degradation rate of polymeric biomaterials by incorporating polymer-degrading enzymes on the biomaterials using different immobilization methods. The inclusion of highly sensitive molecules, such as enzymes, into polymeric biomaterials depends very much on the conditions used during the processing of each material, which are normally not "friendly" (high temperatures and shear rates, organic and acid solvents). This limitation may be overcome by the progress made in protein and genetic engineering fields, where enzyme activities (thermal and pH stabilities, substrate specificity) can be redesigned and tailored to have specific properties. This opens new possibilities of incorporating different biocatalysts during the processing of biomaterials that will then control their degradation profile, allowing for novel and challenging biomedical applications.

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Part II

Production of Biomimetic Coatings on the Surface of Degradable Polymers

13 Bonelike Apatite Coatings Nucleated on Biodegradable Polymers as a Way to Induce Bone Mineralization: Current Developments and Future Trends

A. L. Oliveira and Rui L. Reis

CONTENTS

13.1 INTRODUCTION

Bone is one of the most wonderful examples of nature's ability to engineer living materials by combining different organic/inorganic materials into complex shapes with a controlled specific function. This highly organized mineralized tissue consists of a hierarchy of collagen-based microstructures in association with cartilage and connective tissue. Therefore, when considering an ideal material to replace and mimic bone, synthetic calcium phosphates (currently designated as CaP's or "apatites") can be an obvious answer, since they can replicate the structure and composition of bone mineral — hydroxylapatite (HA) — in a reproducible way.

In the 1970s, CaP's (mostly HA) were synthesized, characterized, and applied in the biomedical field.[1–4] Since then, the interest in these materials has increased, mostly in dental and bone-related applications, but the field has simultaneously become more complex to the everyday user, since

TABLE 13.1
Main Calcium Phosphate Compounds

	Chemical Formula	(Ca/P) Molar Ratio	Abbreviation
	Precipitated CaP		
Dicalcium phosphate	$CaHPO_4$	1.00	DCP
Monocalcium phosphate monohydrate	$Ca(HPO_4)_2 * H_2O$	0.50	MCPM
Dicalcium phosphate dihydrate (Brushite)	$Ca(HPO_4)_2 * 2H_2O$	1.00	DCPD
Octacalcium phosphate	$Ca_8H_2(PO_4)_6 * 5\ H_2O$	1.33	OCP
Precipitated hydroxyapatite (tricalcium phosphate)	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ $0 \le x \le 2$	1.50–1.67	PHA
Amorphous calcium phosphate	$Ca_3(PO_4)_2 * nH_2O$ n = 3–4.5; 15–20% H_2O	1.50	ACP
	High-Temperature CaP		
Monocalcium phosphate	$Ca(HPO_4)_2$	0.50	MCP
α-Tricalcium phosphate	α-$Ca_3(HPO_4)_2$	1.50	α-TCP
β-Tricalcium phosphate	β-$Ca_3(PO_4)_2$	1.50	β-TCP
Sintered hydroxylapatite	$Ca_5(PO_4)_3OH$	1.67	HA
Oxyapatite	$Ca_{10}(PO_4)_6O$	1.67	OXA
Tetracalcium phosphate	$Ca_4(PO_4)_2O$	2.00	TetCP

Data from Bohner, M., *Injury — Int. J. Care Injured,* 31, S37, 2000; Driessens, F.C., in *Bioceramics: Materials Characterizations versus In Vivo Behaviour*, Ducheyne, P. and Lemons, J.E., Eds., Academy of Sciences, New York, 1988; Heughebaert, J.C., in *Biological and Biomechanical Performance of Biomaterials*, Christel, P., Meunier, A., and Lee, A.J.C., Eds., Elsevier Science Publishers, Amsterdam, 1986.

only subtle differences in composition and structure of CaP compounds may have a profound effect on their *in vivo* behavior.[5]

Most calcium phosphates previously used *in vivo* have been calcium orthophosphates, i.e., they contain the orthophosphate group PO_4^{3-}. Two different categories of CaP's should be distinguished[6]: (1) CaP obtained by precipitation from an aqueous solution at or around room temperature (low-temperature CaP), and (2) CaP obtained by a thermal reaction (high-temperature CaP). All calcium phosphate cements belong to the first category, whereas most other CaP products belong to the second category. Table 13.1 summarizes the main calcium phosphate compounds according to each category. The first six compounds precipitate at room temperature in aqueous systems, and the last six are obtained by thermal decomposition or thermal synthesis.

The most important property of CaP's is probably their solubility in water since it allows predicting the *in vivo* behavior of CaP's to a large extent. If the solubility of a CaP is less than the mineral part of bone, it degrades extremely slowly if at all. If the solubility of a CaP is greater than that of the mineral part of bone, it is degraded. The solubility of CaP is inversely proportional to the Ca–P ratio.[9] At body temperature, only two calcium phosphates are stable in contact with aqueous media, such as body fluids.[6,10–12] At pH < 4.2, the stable phase is dicalcium phosphate or brushite ($CaHPO_4 \cdot 2H_2O$), whereas at pH ≥ 4.2, the stable phase is $Ca_{10}(PO_4)_6(OH)_2$ (HA). All the high-temperature calcium phosphate phases interact with water or body fluids at 37ºC to form HA.[10]

It is clear that the material that forms the mineral component of bone should represent the ideal ceramic for bone replacement. In fact, HA presents an excellent biocompatibility and bioactive behavior, which are the mechanical properties dependent on the method of preparation.[13,14] In fact, HA materials have been used in many forms for non-load-bearing applications.[12,13] It is important to distinguish between precipitated hydroxylapatite (PHA) and high-temperature hydroxylapatite (HA) (see Table 13.1). The first is obtained by precipitation in an aqueous solution and is normally poorly crystalline.[6] The molar Ca-P ratio can vary between 1.50 and 1.67 and resembles the mineral

of bone. PHA with a Ca-P molar ratio of 1.50 is often called calcium-deficient hydroxylapatite (CDHA) or tricalcium phosphate (TCP). The latter name has led to confusion with β-TCP, which has almost the same chemical composition but a different crystalline structure.[6] The abbreviation HA usually refers to the hydroxylapatite obtained by thermal treatment, typically above 800ºC. Due to the thermal treatment, HA is crystalline, stoichiometric, and less soluble than the mineral part of bone.[13,14]

Despite having a similar composition and chemistry, the mechanical properties of CaP's are far from being close to those of human bone, which limits their use for load-bearing applications. In fact, CaP's are too stiff and very brittle.[15] The fact is that today's solutions of materials for bone replacement are still far from ideal, with metallic implants still the first choice for load-bearing applications, despite all the problems associated with stress shielding and long-term application. Although progress is currently being made, the right balance between surface and bulk properties of a material is still to be achieved. For the near future, one of the most interesting solutions is to use CaP's with ideal bone tissue growth effects as coatings on the surface of load-bearing materials.[8,13,16–20] CaP's disclose osteoconductive properties allowing for the formation of bone on their surface by attachment, migration, proliferation, and differentiation of bone-forming cells.[19,21] Therefore, despite their low mechanical properties, these types of coatings have great potential for bone fixation applications or on scaffolds for tissue engineering. In fact, these coatings can be tailored in terms of chemical composition, crystallinity, and resorbability[22,23] and also can be loaded with osteogenic biological molecules[24–26] or serve as beds for the seeding of living cells that will stimulate bone formation.

At present, the commercially available methods to produce such CaP coatings are few and typically still the same as those proposed a decade ago, having several disadvantages such as difficulties in controlling the calcium phosphate layer composition, resorbability, weak adhesion to the substrates, the use of high temperatures, and the cost of the process. Moreover, these methodologies are not effective on coating complex shapes. On the other hand, new challenges are now being raised. The ability to coat the interior of a porous material would be highly interesting when thinking about the fashionable concept of tissue engineering. These coatings would facilitate the cell attachment and proliferation in the interior of the scaffold, followed by the process of vascularization. For the last 30 years, there has been a great amount of research in this particular area to face the need for effective coating methodologies.

13.2 EVOLUTION OF THE COATING METHODOLOGIES

In the history of bone replacement, various orthopedic implant materials have been introduced, particularly metallic materials such as stainless steel or titanium alloys.[27] Because a stable fixation of these implants to the bone was found to be critical to the long-term stability in applications such as joint replacement, an input was made for the development of different CaP coating methodologies to induce bone-bonding ability and therefore to create more stable interfaces.[15,28]

The plasma-spraying technique is, to date, the major commercially available method, used for coating CaP on metallic implants.[29–32] The first coated implant was commercialized in 1980 by Valen for dental applications. It is outstanding to imagine that the same technique is still being used, if we think about how much we have progressed since then (How would our lives be if we were still using computers from that time?). But the fact is that reproducibility and economic efficiency of the process are advantages that made it famous from 20 years ago to today.[30,31] However, this method presents some crucial drawbacks affecting the long-term performance and lifetime of the implant. The most significant are the poor coating–substrate adherence[31] and lack of uniformity of the coating in terms of morphology and crystallinity.[32,33] Since plasma-spraying is a high-temperature and line-of-sight process, there are also some aspects that were not solved yet, such as the deteriorating effect of intense heat on substrates, nonuniformity in coating density, wide range of band strength, and limitation in coating implant devices with complex shapes.[31–33]

Other studied approaches have been sputtering coating techniques that are able to increase the bond strength between the coating and the substrates.[34–36] However, the drawback inherent to this technique is that the deposition and the process itself are very slow. By using a magnetically enhanced variant of radio-frequent sputtering, this problem can be solved, but the endurance and the Ca-P ratio of the coating require further *in vitro* and *in vivo* studies before this technique can be applied routinely to produce crystalline pure CaP ceramic coatings on implant surfaces.[10] A collection of other methodologies have been proposed such as dip coating sintering,[37] chemical vapor deposition,[38] sol-gel deposition,[11,39] ion implanting,[40] laser deposition,[41–43] laser cladding,[44] and electrochemical processes such as electrophoretic deposition,[45] electrocrystallization,[46] and anode oxidation.[47] Despite all the investigations carried out, the produced coatings can suffer from at least one of the following problems (which differ from method to method)[17]: lack of coating adherence to the substrate, thickness nonuniformity, poor structural integrity, and nonstoichiometric composition of the coatings. In fact, each of the aforementioned techniques has its own technical limitations, and so far, an optimal technique for producing physiologically stable and interfacially adherent apatite coatings has yet to be developed. Thus, there is a demand to develop new methods able to face new challenges, such as forming an apatite layer with properties similar to those of bone calcium phosphates on the surface of new emerging materials and on various complex-shaped materials that is capable of enhancing biocompatibility as well as bioactivity, when engineering bone implants or designing tissue engineering scaffolds. On the other hand, the ability to incorporate bioactive agents in these coatings can be a very promising therapeutic strategy for a site-specific delivery system for stimulating bone tissue regeneration.

13.3 BIO-INSPIRED COATINGS

The CaP minerals found in natural hard tissues are produced spontaneously in a physiological environment at low temperatures from moderately supersaturated mineralizing solutions.[9] In the recent years, there has been an increasing interest in the natural formation of CaP's as a way to learn, understand, and apply natural processes for producing CaP coatings that are biologically identical to bone apatite. The so-called biomimetic preparation of calcium phosphate coatings on implant materials has emerged as a new concept. Rapid progress has been made in the development of these coatings, and several methodologies have been proposed. This type of approach is particularly suitable to coat polymeric materials,[16,49] as it can be carried out at low-temperature reaction conditions.

13.3.1 The Biomimetic Methodology

A calcium phosphate coating was first grown on a substrate by a biomimetic process by Kokubo et al.[50] in 1990 using bioactive CaO-SiO_2-based glass particles that were set in contact with the substrates to induce apatite nucleation on their surface in a simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma at body temperature. This solution was developed also in 1990 by the same author.[50] Silicate ions containing silanol groups (Si-OH) are supposed to be released from the bioactive glass and adsorbed on the substrate surface to induce apatite formation. After 1 week (the time established for apatite nuclei to form a layer), the apatite grew subsequently by immersing the substrates in 1.5 SBF with ion concentrations 1.5 times higher than those of SBF. By this method, an apatite layer has been coated on ceramics,[49,51] metals,[49,51] and polymers,[49,51] such as alumina glass, zirconia, titanium, polyethylene, polymethylmethacrylate, ethylene–vinyl alcohol copolymer, polyethylene terephthalate (PET), poly(ethersulfone) (PES), polyamide (PA 6,6), poly(vinyl alcohol), and silicone. It was also shown that this apatite layer could be formed not only on flat surfaces but also on curved surfaces, of small particles, long fibers, and woven cloths of various materials.[48,51]

The adhesion strength of the apatite layer to the polymeric (or other) surfaces by this process was, however, not sufficient for clinical applications.[48] Surface modifications were then applied, prior to the biomimetic treatment, to improve the adhesion strength by increasing the number of polar groups on the substrate surface.[52–55] These groups were found to act as favorable nucleating sites for apatite formation in the surface of the polymers. Various methods for surface modification of different polymers were reported by Kokubo's group. Chemical treatments with sodium hydroxide (NaOH)[52] or (hydrochloric acid) HCl[53] solutions before biomimetic coating have shown to improve the adhesion strength and to reduce the induction periods for apatite formation, being strictly dependent on the type of polymer. Surface modifications induced by a glow-discharge treatment[54] and ultraviolet irradiation (UV)[55] were also tested for the same polymers and similar effects were described.

An adaptation of the biomimetic methodology was also used by Reis et al.[56] in which the samples were rolled on a bed of wet bioactive glass particles before immersion in an SBF solution. The methodology was effective in coating different types of polymers and shapes such as a high-molecular-weight polyethylene,[56] a biodegradable starch poly(ethylene vinyl alcohol) blend (SEVA-C), and a polyurethane foam.[56] Nevertheless, the same problem associated with a lack of coating adhesion was also observed. Therefore, different surface modifications are presently being experimented with, some of which have already led to some results. Surface treatments such as potassium hydroxide (KOH), UV radiation, and overexposure to ethylene oxide sterilization, on SEVA-C substrates, before the biomimetic process have proved to be very effective in increasing the adhesion to the substrate and reducing the incubation periods for apatite formation.[57]

Another way of tailoring the properties of the apatite layer formed by the herein described biomimetic methodology is by the side of the solution, i.e., changing the composition of the SBF.[58,59] Kim et al.[59] have reported that different apatite layers can be produced on polyethylene terephthalate (PET) substrates in solutions where the ion concentrations were changed from 0.75 to 2.00 times those of SBF. Increasing the ionic activity product has then resulted in lower Ca-P ratios of the apatites. The same authors[58] have also reported that by increasing the carbonate ion content, apatites with composition and structure nearly identical to those of bone apatite could be produced. In fact, this solution is known to be deficient in relation to the HCO_3^- content when compared with human blood plasma.[58,60] Therefore, this group is now proposing a new revised SBF (R-SBF) with an ion composition closer to the human blood plasma (higher amounts of HCO_3^-), to replace conventional SBF.[60]

13.3.2 Other Premineralization Routes

There is an enormous amount of published work[55,61–65] using other different biomimetic routes for the formation of apatite layers on the surface of different materials. Some of them are based on surface modifications, by chemical and physical means that are claimed to induce direct bioactivity in the surface of the materials.[63–65] Other methodologies use nucleating agents to induce the formation of the bioactive layer.[55,62]

Bonelike apatite was successfully formed on organic polymers by a biomimetic process using sodium silicate solution as a nucleating agent, instead of bioactive glass particles, as reported by Miyaji et al.[62] A dense apatite layer was formed not only on limited surfaces but also on whole surfaces of fine PET fibers constituting a fabric. Therefore, this method enabled the production of apatite coatings on various materials with complex shapes. The "traditional" biomimetic process is not so effective for coating materials with complex shapes, since the apatite nuclei are formed only on the material surface that is facing the glass grains.[62] On the other hand, it was also possible to reduce the incubating periods for apatite formation to only 6 hours.[62]

The grafting technique has also become very popular as a way to immobilize functional groups at the surface of polymers that can encourage apatite formation. For instance, Oyane et al.[66] developed a different methodology in which ethylene–vinyl alcohol copolymer (EVOH) substrates

were modified by grafting silane coupling agents at its surface, in order to produce silanol groups, such as tetraethoxysilane (TEOS). Their apatite-forming ability was examined in SBF and 1.5 SBF. Only in the latter case was it possible to observe the formation of an apatite layer after 21 days.[66] Nevertheless, the Ca-P molar ratio of the apatite formed in 1.5 SBF was much lower than that of the apatite in the natural bone. Therefore, the same authors[67] tried also to incorporate calcium ions in the silicate phase, by using a calcium silicate solution prepared with the coupling agent. The release of these ions accelerated the apatite nucleation by increasing ionic activity product of the apatite locally near the surface.[67] Recently, Kim et al.[63] proposed a different methodology to incorporate silanol groups at a polyethylene substrate via photografting of vinyltrimethoxysilane and hydrolysis. The substrate modified in this way formed a dense and homogeneous bone-mineral-like apatite layer in a 1.5×SBF solution, after 7 days.

Tretinnikov et al.[61] also developed a bioactive polymer by using surface modification by grafting to immobilize organic compounds. An organophosphate polymer was chemically bound onto a polymer film by surface graft polymerization of a phosphate-containing monomer.[61] As the phosphate group is one of the building blocks of HA and has a high affinity toward calcium ions, polymeric materials modified by surface-grafted water-soluble organophosphate polymer induce bone deposition of Ca and PO_4 ions in the form of a hydroxy carbonate apatite (HCA) layer.[61] It is then expected that covalent immobilization of organophosphates will open the way for developing bioactive bone-bonding polymers.

More recently, Oliveira et al.[68] have used plasma and chemically induced graft polymerization of different acrylic hydrophilic monomers on starch-based blends, in order to improve the cell adhesion and proliferation. After this treatment, the materials were incubated in a solution of calcium chloride ($CaCl_2$). Subsequent immersion in an SBF solution led to the formation of an apatite layer after 15 days, for all surface modifications. Furthermore, these treatments were also effective in improving the cell adhesion and proliferation with respect to the original starch-based blends.

Another approach was used by Mucalo et al.[64] to coat apatite in cotton substrates, by grafting phosphate groups at the surface of the substrates using a phosphorylation methodology and subsequent SBF immersion. Yokogawa et al.[65] found that soaking in $Ca(OH)_2$-treated phosphorylated chitin fibers also led to the deposition of an apatite layer, after SBF immersion.

The Langmuir Blodgett (LB) technique, another very interesting approach, is a type of supramolecular assembly that can be used to produce an organic template with a specific head group for the nucleation of calcium phosphate crystals.[63] The LB process produces a thin organic film with a very organized structure, fulfilling the requirements of an organic template for a controlled mineralization process. Costa and Maquis[69] investigated apatite formation in SBF using a ω-tricosenoic acid monolayer film that was prepared by the LB method, using calcium carboxylate as a functional group. They reported that the nucleation of apatite was induced by carboxyl groups that reduced the interface energy between the nucleus and substrate. The morphology of the apatite crystals grown was strongly affected by the structure of the monolayer. It was further indicated that the crystal orientation between the apatite and organic material took place *in vitro*, possibly due to the similar mechanisms observed in the biogenic materials.[69]

Besides the use of SBF as a mineralizing environment, other biomimetic solutions have been developed for the formation of an apatite layer. For example, Taguchi et al.[70] developed an apatite formation process using hydrogels — an alternate soaking process — to form large amounts of apatite in SBF for a considerably short period of time (42 hours). The soakings have alternated every hour between $CaCl_2$ and dihydrogen phosphate (H_2PO_4) solutions. The same methodology was also effectively applied by Furuzono et al.[71] to coat a silk fabric proposed as a biomaterial for bone replacement. Nevertheless, this methodology does not seem very practical since it requires a constant change of solutions every hour. Kim et al.[72] have used a simple method of coating thin films of low crystalline apatite crystals by using a filtrated solution containing calcium and phosphate ions to coat a poly(lactide-co-glycolide) copolymer sponge (PLGA). The material was coated by this process within 24 hours. The work of Yuan et al.[73] indicates that an apatite layer was formed

in the surface of poly(*L*-lactic acid) (PLLA) after 2 weeks without the aid of a nucleating agent, by immersing the substrates in a solution inspired in SBF but with 1.5 times its ion concentration. Rhee and Tanaka[74,75] have shown that an apatite layer could be formed in the surface of a collagen membrane after 1 week with the same 1.5×SBF solution, but to which citric acid was also added. This same solution is able to induce the formation of an apatite layer, after the same period, in the surface of a cellulose cloth.[76] The results therefore suggest that citric acid has a nucleating ability and can accelerate the nucleation of apatite on the nonbioactive studied substrates.

Recently, a new alternative methodology was proposed by Leonor and Reis,[77] through a novel autocatalytic deposition process. The developed coating methodology uses a deposition route that is totally "electroless," i.e., does not require the use of electric current application for its application, being based on redox reactions. It was possible to produce a CaP coating on the surface of three types of polymers (HMWPE, SCA, SEVA-C). Two types of solutions, "alkaline" and "acid" baths, were studied to produce the novel proposed autocatalytic CaP coatings. With these solutions, well -adherent apatite coatings could be formed on the surface of both biodegradable and bioinert polymers.[77] The developed route seems to be a very promising and simple methodology for use as a preimplantation treatment to coat several types of materials prior to their clinical application.

Herein are described some examples that illustrate the variety of possibilities that can be explored when using or developing a biomimetic approach. All these surface modifications and premineralization routes previously described can play an important role in enhancing the biocompatibility and bioactivity of a biomaterial, particularly polymers. In fact, cells are sensitive to several surface properties such as roughness, energy, chemistry, and even more subtle characteristics such as relative crystallinity.

It is possible to find several advantages of the biomimetic approach over the methodologies previously described. In fact, a biomimetic coating is expected to show higher bone-bonding ability due to its similarity to the mineral of bone. On the other hand, the adhesion to the substrate can be enhanced by means of several methodologies or by different surface pretreatments. Tailored apatite coatings with different Ca-P ratios and crystallinities are also possible to be obtained. Another very important advantage is that no adverse effect of heat on substrate occurs, since these methodologies work at operating temperatures that allow them to be applied to a range of different materials such as biodegradable polymers. It is also the simplest and more cost effective of the approaches available to create a biological-like apatite layer. Taking advantage of all this, different biomimetic approaches are being developed by our group to be able to effectively coat a range of bioinert and biodegradable polymers processed into a large variety of shapes for bone-related applications.

13.4 COATING STARCH-BASED BIODEGRADABLE POLYMERS

Bonelike calcium phosphate coatings have great potential when applied to biodegradable polymers since they can stimulate the tissue regeneration at the bone–implant interface while the material is being resorbed (ideally at the same rate that bone is being formed). To achieve this ideal synchronized behavior, with the stress slowly transferred from implant to bone, has been the main goal for scientists in this area.

Starch-based polymers are particularly interesting for bone-related applications (replacement, tissue engineering scaffolding, etc.). Besides being biodegradable, inexpensive (when compared with other biodegradable polymers such as polylactic acid), and available in large quantities,[78–80] starch-based polymers can be converted into complex geometries that exhibit interesting mechanical properties, by using standard equipment developed for the processing of synthetic polymers or by means of using distinct innovative methodologies. Furthermore, in addition to their processing versatility, they exhibit a biocompatible behavior, already demonstrated by *in vitro*[81–83] and *in vivo* studies.[84] Therefore, they are under consideration for a wide range of biomedical applications such as bone replacement/fixation,[85,86] novel hydrogels and partially degradable bone cements,[87,88] drug delivery carriers,[88,89] or temporary scaffolds for tissue engineering applications.[89,90]

In the case of bone-related applications such as tissue replacement/fixation or tissue engineering scaffolds to be applied in load-bearing sites, these systems must exhibit mechanical properties that match those of human bone, associated with degradation kinetics adequate to the healing of the tissues to be replaced or fixed.[91] It was reported by our group that the physical and mechanical properties of these materials could be optimized by controlling the morphologic developments within the molds, using nonconventional processing routes.[92,93] On the other hand, the incorporation of bonelike inorganic fillers, such as hydroxylapatite (HA)[94,95] or bioactive glasses,[96] is another interesting approach, allowing for the development of degradable composites that can combine an attractive range of mechanical properties with the desirable bone-bonding behavior. Since the essential condition for materials to bond to living bone is the formation of a biologically active bonelike apatite layer on their surfaces, different coating methodologies are also being developed through biomimetic processes for producing such types of layers on the proposed materials.[56,57] Finally, a novel emerging application is also currently being proposed, on which starch-based polymers would serve as temporary scaffolds for the transplanted cells to attach, grow, and maintain differentiated functions in a range of tissue engineering applications.[89,90] In fact, it was already possible to develop distinct porous architectures based on these starch-based biodegradable blends[89,97] by means of using different and innovative processing routes either based on melt-processing technologies[97] or on microwave baking[89] and subsequently to produce biomimetic coatings on these materials that are aimed not only at enhancing cell adhesion and proliferation, but also at tissue ingrowth.

13.4.1 Sodium Silicate as a Precursor for a CaP Coating

As mentioned before, when considering materials with complex shapes, such as porous 3-D architectures, the traditional biomimetic methodology is not so effective. This can be explained by the considerable difficulty in forming an apatite coating, since the apatite nuclei will preferentially grow in the surfaces that are facing the bioactive glass particles. In order to overcome this obstacle, a new biomimetic methodology to produce bioactive coatings on the surface of starch-based or other polymeric biomaterials is being proposed by Oliveira et al.,[98] using a sodium silicate gel as an alternative nucleating agent. Sodium silicate gel can reach inside the pores of porous 3-D architectures to be used on tissue replacement and in tissue engineering scaffolding. This new methodology is aimed at[98] (1) reducing the incubation periods, (2) improving of the adhesion strength between the coating and substrate, (3) being able to coat the inside of pores in porous 3-D architectures to be used on tissue replacement and as tissue engineering scaffolds, and (4) producing CaP layers with different (tailored) Ca-P ratios.

The studied materials included typically different starch-based blends processed into different shapes. To produce the bioactive coatings, the materials were "impregnated" with a commercially available sodium silicate gel, which acted as an alternative CaP nucleating agent. After the sodium silicate treatment, the samples were soaked in a simulated body fluid (SBF) at 37ºC. After 7 days, the ion concentration of SBF solution was raised to 1.5× in order to make the apatite nuclei grow. The procedure is schematized in Figure 13.1.

Figure 13.2 shows the water-uptake versus time for SEVA-C compact and porous structures, untreated and treated with sodium silicate.

For compact structures, the equilibrium hydration degree of untreated samples is about 25%, after 50 hours. This hydrophilic behavior is mainly a result of the presence of starch and vinyl alcohol hydroxyl groups, as it has been previously described.[57,99] Untreated porous structures achieve the equilibrium hydration degree, around 45%, after the first 10 hours of water uptaking. This higher hydrophilicity may result from the existence of high amounts of polar groups available in the structure after reaction of hydrogen peroxide with starch. On the other hand, porous structures have a higher specific surface than compact ones, which allows for a higher water uptake. With sodium silicate treatment, the amount of water taken up increases around 5% in both types of

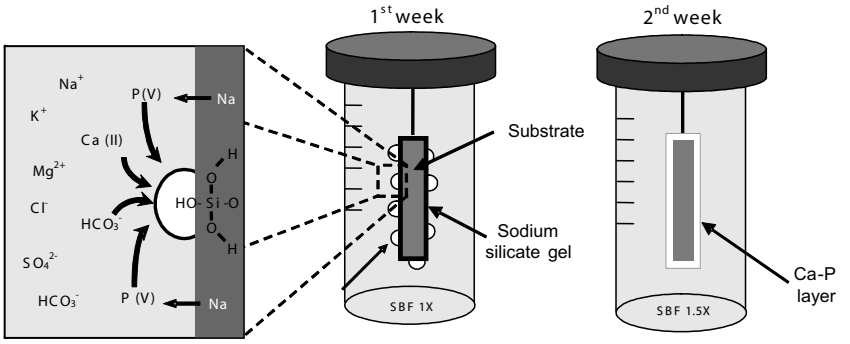

FIGURE 13.1 Schematic representation of the sodium silicate gel biomimetic methodology. Adapted from the procedure developed by Kokubo et al.[50,51] and adapted by Reis et al.[56,98]

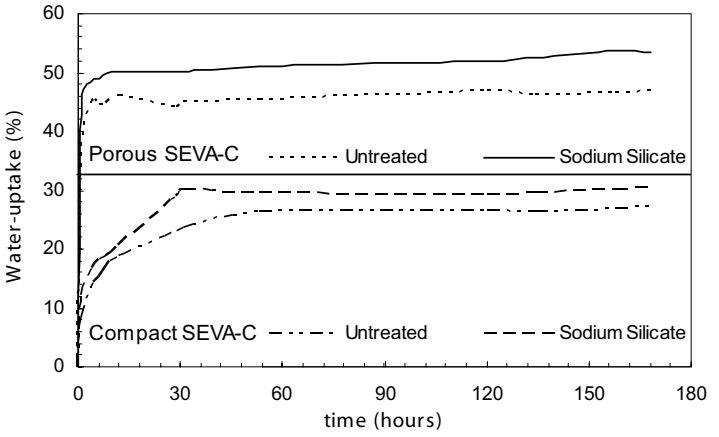

FIGURE 13.2 Water uptake (%) versus time for untreated and sodium silicate-treated compact and porous SEVA-C samples.

structures, this observation being more significant in the case of the compact material. The materials used as controls (compact HMWPE and porous PU) did not uptake water after sodium silicate treatment (data not shown).

13.4.1.1 Apatite Formation

After immersion in SBF for several periods, the formation of an apatite layer was studied. Figure 13.3 shows the SEM photographs of the evolution of the typical films formed on the surface of the treated materials.

In sodium silicate-treated materials, after only 6 hours of immersion in SBF, it was possible to observe the formation of very cohesive apatite-like layers that became fragmented due to the swelling of the polymers (data not shown).[98] For the porous materials, the apatite layer could also be observed inside the pores, clearly covering the cell walls. When comparing with the traditional biomimetic treatment, the latter was not so effective at reaching the bulk of porous structures. This result is very promising for the development of cancellous bone replacement materials and for precalcifying bone tissue engineering scaffolds. For the SEVA-C compact material, the corresponding Ca–P ratios for the apatite formed in this earlier stage of nucleation is around 1.5, which is typical of tricalcium phosphate (TCP). After 30 days, the morphology of the layers tended to develop the so-called cauliflower morphology,[56] which is clearly shown in both compact and porous

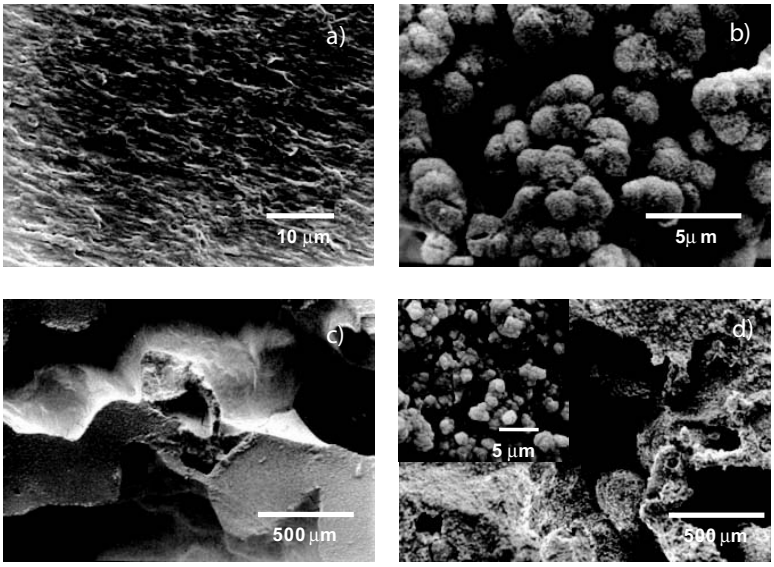

FIGURE 13.3 SEM micrographs of compact SEVA-C treated with (a) sodium silicate and after (b) 30 days of immersion in SBF and porous SEVA-C treated with (c) sodium silicate and after (d) 30 days of immersion in SBF. (From Oliveira, A.L. et al., *Biomaterials,* 24, 2575, 2003. With permission.)

structures. On the other hand, the respective Ca–P ratios are very close to the value attributed to hydroxyapatite (1.67). The biomimetic methodology proposed in this case led to an increase in the water-uptake ability of the polymers (Figure 13.1), allowing the materials to absorb higher quantities of Ca^{2+} ions from the SBF solution. As a consequence, the Ca^{2+} ion concentration in the surface will be increased, leading to the formation of additional nucleating sites for the CaP coating formation. On the other hand, the increase of the surface hydrophilicity (data not shown),[98] raising the number of polar groups in the surface, could contribute to the formation of silanol groups that are well-known apatite inductors. This theory has been previously described by Kokubo et al.[16,48,50] Figure 13.4 presents the TF-XRD patterns of the surface of compact (Figure 13.4a) and porous (Figure 13.4b) SEVA-C, before and after immersion in SBF.

TF-XRD spectra exhibited the formation of a partially amorphous CaP film with the crystalline peaks mainly corresponding to hydroxylapatite, for the longer SBF immersion periods. On materials used as controls (compact HMWPE and porous PU), it was not possible to observe apatite formation (data not shown),[98] indicating that this methodology is highly adequate only for materials that have a strong swelling ability. In this case, even with the corresponding difficulties associated with biodegradable polymers raising from continuous pH and surface changes as a function of time, the methodology was successful in generating CaP coatings.

13.4.1.2 Cell Adhesion

Figure 13.5 shows the SEM micrographs of the morphology of SEVA-C surface after immersion in SBF for 15 days and subsequent SaOs-2 cell culturing for the first 24 hours.

In the presented figures, it is possible to observe that the apatite layer is formed by aggregates of nucleus that have grown into the previously described cauliflower morphology,[56,57] after 15 days of immersion in SBF (please remember that the SBF concentration was raised to 1.5 times after 7 days).

Of interest to the study of cell adhesion, it is possible to observe, on the same SEM micrographs (Figure 13.5a and 13.5b), that these preliminary results indicate good cell attachment to both types of coated surfaces. In fact, the crystallinity of a biomaterial surface is known to determine specific

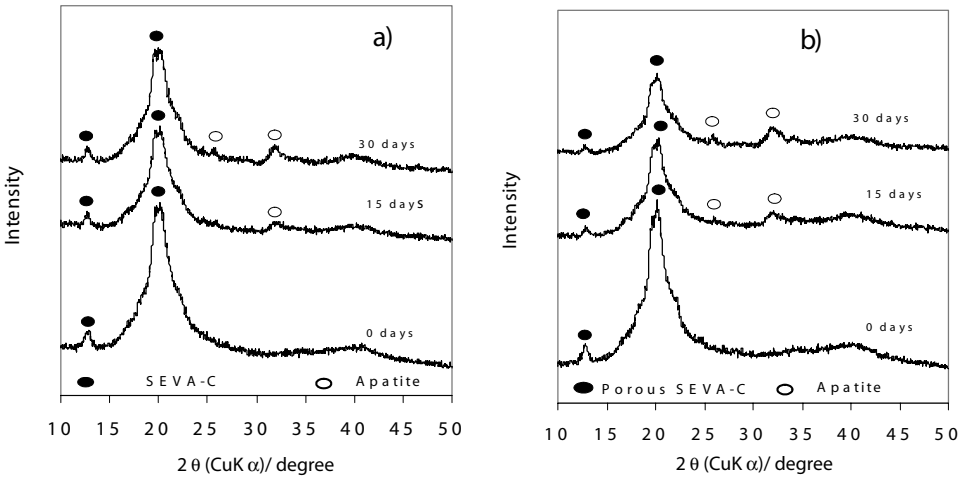

FIGURE 13.4 Thin-film x-ray diffraction spectra of the films formed after treatment with sodium silicate. (a) Compact and (b) porous SEVA-C after 0, 15, and 30 days of immersion in SBF. (From Oliveira, A.L. et al., *Biomaterials,* 24, 2575, 2003. With permission.)

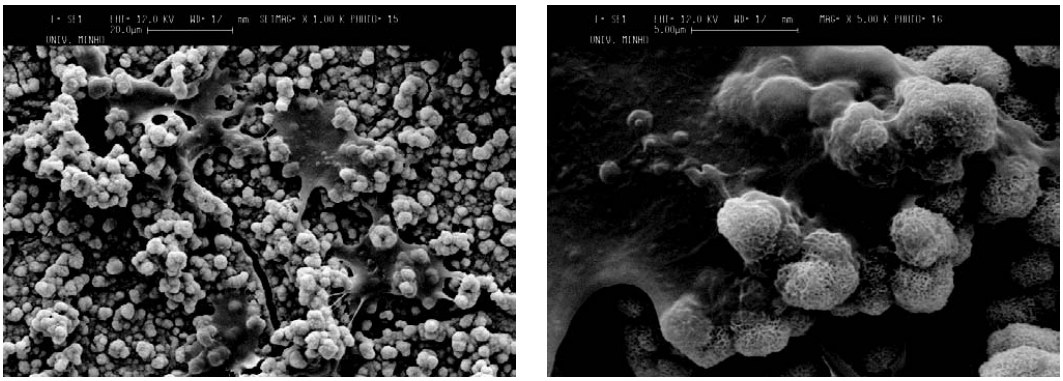

FIGURE 13.5 SEM micrographs showing the morphology of the apatite coatings formed on the surface of SEVA-C and the typical aspect of the SaOs-2 cells adhered to it after 24 h of cell culturing.

cell responses such as the organization of cytoskeleton filaments and cell proliferation mechanisms.[100,101] Furthermore, it can be said that SBF treatment — or more specifically, the obtained characteristics of the coatings — influence positively cell proliferation kinetics (data not shown).[102] In this study, cell behavior seems to depend on the coating crystallinity being slower for more amorphous surfaces.

13.5 NEW OPPORTUNITIES FOR COATED MATERIALS

One of the most promising characteristics of several biomimetic methodologies is that they can be suitable to produce apatite coatings onto complex-shaped materials.[16,56,103] As a result, there are new opportunities for these types of coatings in the field of tissue engineering as a way to enhance the formation of bone tissue. Some possibilities are presently being considered, based on the incorporation of osteogenic biological molecules in the biomimetic coatings.[24,26,104,105] Due to the physiological coating conditions presented by some methodologies, it is expected that these bioactive factors can preserve their biological activities. Therefore, considering the slow but definite

degradation of such coatings,[9,106–108] they may be very suitable to serve as carriers for these molecules. The options for creating a delivery system in these coatings are numerous.

Certain molecules, such as protein growth factors, can regulate various cell functions such as growth, differentiation, secretion, and apoptosis.[109] Bone morphogenic proteins (BMPs) are an important class of bone growth factors that might revolutionize how clinicians treat such diverse orthopedic applications as the healing of broken bones or increasing bone density lost through aging.[109–111] Murphy et al.[105] developed a system for sustained release of a bioactive vascular endothelial growth factor from poly(lactide-co-glycolide) scaffolds. These scaffolds were previously mineralized in an SBF solution.[112] For example, de Bruijn et al.[113] reported that by a co-precipitation process it was possible to incorporate a bone morphogenic protein, rhBMP2, in an apatite coating on a Ti alloy substrate.

A more biologically driven approach involves the addition to the apatite layer of living osteogenic cells to create an autologous bone-graft substitute. Due to the similarity of the formed layers with bone mineral, they represent friendly surfaces for cell attachment and proliferation, which subsequently will stimulate the formation of bone tissue either *ex vivo* or after implantation. Studies developed by de Bruijn et al.[113] show that rat-, goat-, and human-derived bone marrow cells can be expanded *in vitro* and maintain their osteogenic potential *in vitro* and *in vivo* when grown under specific osteogenic culture conditions. These results indicate that new developments, such as those in bone tissue engineering, are promising to yield a whole new generation of osteoinductive implant coatings derived from patients' own tissues.

13.6 CONCLUSIONS

A number of biomimetic coating routes are presently being developed in order to produce CaP layers on orthopedic implants and tissue engineering scaffolds.

The CaP minerals in natural hard tissues are formed in a physiological environment at low temperatures from moderately supersaturated mineralizing solutions. Therefore, in recent years, there has been an increasing interest in the so-called biomimetic preparation of calcium phosphate coatings on implant materials, in contrast to the major commercially available method in use nowadays, which is still the plasma spraying technique. Rapid progress has been made in the development of these bio-inspired coatings, and several methodologies have emerged. This approach is particularly suitable for coating polymeric materials (as it can be carried out at low temperature reaction conditions) and complex-shaped scaffolds for tissue engineering. Furthermore, other possibilities are presently being considered based on the incorporation of bioactive factors in the biomimetic coatings, such as osteogenic biological molecules. The herein presented biomimetic methodology, using a sodium silicate gel as nucleating agent, was successfully developed and produced a well-defined calcium phosphate layer on the surface of a starch-based biodegradable polymer. With this treatment, it was possible to reduce the induction period for the formation of a well-defined apatite-like layer. Furthermore, when applied to porous materials, this methodology was highly effective, since a clear apatite-like layer was observed inside the pores, clearly covering the pore walls. It also can be said that the obtained characteristics of the coatings influence positively cell adhesion and proliferation kinetics. This proposed route is quite simple and very promising for developing cancellous bone replacement materials and for precalcifying bone tissue engineering scaffolds.

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14 Biomimetic Coatings, Proteins, and Biocatalysts: A New Approach to Tailor the Properties of Biodegradable Polymers

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CONTENTS

14.1 INTRODUCTION

As the average age of the population increases, there is a growing demand for biomedical materials that will survive for 10 to 20 years longer than today's generation of biomaterials. The greatest

promise for achieving extensive improvements in long-term clinical repair of the skeletal system is to concentrate research and thinking on creating a new generation of biomaterials that enhances the body's own repair mechanisms, i.e., regeneration of tissues.[1]

An orthopedic surgeon described[2] the ideal implant as "a material that performs its function without toxicity or foreign body reaction, and then is completely resorbed and replaced by new tissue." It is now well established that no material implanted in living tissues is inert.[3,4] In fact, all materials elicit a response from living tissues.[3,4]

Typically when an artificial implant is implanted into a bone defect, it is encapsulated by noncalcified fibrous tissue, to be isolated from the surrounding bone, and the bone is not adherent to the implant, which results in small gaps between the natural bone and implant, leading to the movement at the implant–tissue interface.[5] Eventually, this causes the failure of the device and the need for additional surgeries to replace the loose implant.[5] As a consequence, a stable interfacial bond between tissue and implant must be achieved in order to obtain the survivability of an implant and an equivalent physical response. On the other hand, the production of a stable interface is dependent on controlled physical stimulus.[3,4]

In the last three decades, a new class of biomaterials designated as surface-active biomaterials[3] has become more widely used in clinical applications, particularly musculoskeletal and dental applications.

These surface-active biomaterials permit the formation of direct physicochemical bonds between bone tissue and the implant without a fibrous capsule formation.[6] The interfacial bond with bone is generally stronger than either the bone or the implant.[3,4] Also, it has often been assumed that interfacial stability achieved with a bioactive fixation would ensure an improvement in implant survivability.[7]

The chemical composition of these substances usually includes phosphorus and calcium and is thus similar to the mineral composition of bone tissue.[4] These materials include dense hydroxyapatite ceramics,[8,9] bioactive glasses,[3,4] bioactive glass-ceramics,[10,11] bioactive composites,[12,13] and bioactive coatings.[9,14]

Proteins, through their unique and specific interactions with other macromolecules and inorganics, control biological structures and functions in organisms.[15] In this sense, the concept of biomimetics may also envisage the use of inorganic surface specific proteins as templating or enzymatic agents for controlling materials assembly.[16] The traditional approach involves protein isolation and purification, amino acid analysis, and sequencing, which may be complex and time-consuming procedures. An alternative approach is to use existing proteins that may be able to modulate the structure of inorganic surfaces, but also to regulate other surface interactions, such as cell attachment, growth, and differentiation, by presenting bioactive proteins to the interface.

This chapter will focus on the role of different biomolecules on biomineralization and how they can be used to produce coatings with tailorable properties for biomedical applications.

14.2 CALCIUM PHOSPHATE CERAMIC

Hydroxyapatite (HA–$Ca_{10}[PO_4]_6[OH]_2$) is one of the calcium phosphates that makes up the majority of the inorganic mineral component of human bones and teeth.[9,17,18] This inorganic phase may include smaller amounts of other calcium phosphates, calcium carbonate, calcium fluoride, calcium hydroxide, and calcium citrate. Natural hydroxyapatite is predominantly crystalline, although it may also be present in amorphous forms, without any crystalline structure but with the same chemistry. In fact, its compatibility with surrounding tissue has been experimentally proved to be superior to any other material, and the physical and chemical properties closely related to *in vivo* phenomena have been well studied.[19] The advantage of using HA as a bioceramic compared to other bioceramics, such as Bioglass® or A-W glass ceramics (Ceravital®), is its chemical similarity to the inorganic component of bone and teeth and its high bioactivity and biocompatibility.[20]

Biological responses, such as bone bonding and the biodegradation or biostability properties of these materials, are very important in clinical applications due to the fact that unlike natural bones, calcium phosphates ceramics are not alive, so they are in constant interaction with different biological and chemical substances inside a human body.[21,22] Biodegradation of any implant is governed not only by material characteristics, but also by conditions of the biological environment. Biodegradation is considered to have a large influence on the bone-bonding properties.[21,22] Between the biological apatite (the mineral phase of calcified tissues, such as enamel, dentine, and bone) and the pure synthetic HA, there are some differences that influence the bonding mechanism. The main differences[17] are that biological apatites contain carbonate and are always calcium deficient — i.e., nonstoichiometric — and often include cations such as Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, and F^{-}. Besides, the degree of crystallinity is always poor with a crystal size lower than 0.2 μm. Also, the biological apatite contains organic phases. These phases exist at the grain boundaries and have a large influence on the physicochemical and biological properties of the apatite.[23]

The bonding mechanism of HA implants differs from the bioactive glasses and glass ceramics, due to the fact that the HA has much lower solubility and does not include a glassy phase or Si ions.[24] The HA forms an indistinct apatite layer when and where bone mineralization occurred.[25,26] So, the apatite formation occurred on the surface of this ceramic at the same time that mineralization occurred in the surrounding tissues.[26] The HA is much less soluble than A-W glass-ceramic (Ceravital®),[10,11] though it may offer preferable sites for apatite precipitation without hydrated silica. Therefore, it is speculated[26] that the HA does not increase the Ca^{2+} ion concentration and that apatite precipitation on the ceramic does not begin until the surrounding environment becomes appropriate for bone mineralization or apatite formation.

14.3 PROTEINS AND ENZYMES: GENERAL PROPERTIES

Proteins are the most abundant organic molecules within the cell extracellular and intracellular medium, where they are responsible for ensuring multiple biological functions such as transport, regulation of pathways, protection against foreign molecules, structural properties, protein storage, and the catalysis of a great diversity of reactions as biocatalysts (enzymes).[27]

14.3.1 Protein Composition and Structure

In a molecular perspective, proteins may be considered as polymeric structures composed by 20 distinct amino acids linked by amide (or peptide) bonds.[27–29] Amino acids are therefore the building blocks of polypeptides and proteins, which consist of a central carbon linked to an amine group, a carboxyl group, a hydrogen atom, and a side chain (R groups). R groups can be classified as nonpolar groups, uncharged polar groups, or charged polar groups; their distribution along the protein backbone renders proteins with distinct characteristics.[28,29]

The structure of a protein is not, however, as simple as a polysaccharide or other polymer. Generally, the protein structure is described in four levels. The primary structure of a protein is its amino acid sequence, whereas the secondary structure refers to the local spatial arrangement of the polypeptide's backbone atoms without regard to the conformation of its side chains. The folding of the polypeptide chain is responsible for putting in close contact different parts of the chain to create binding sites to the substrate, etc. The tertiary structure is related to the three-dimensional structure of the entire polypeptide. When proteins are composed of more than one polypeptide chain (referred to as subunits), the resultant spatial arrangement of its subunits is known as the protein's quaternary structure.[27,29] Many enzymes are globular proteins and consequently their phenotype is a quaternary structure.[27,30]

The configuration assumed by a protein, and thus which determines its properties, is the one that minimizes the molecule's free energy. Protein conformation is determinant for protein bioactivity, being known that a certain three-dimensional structure is essential for a protein to work

properly. Most of the forces that stabilize the protein structure are weak (hydrogen bonding, ionic and hydrophobic interactions, van der Waals forces), giving some flexibility to the macromolecule. In general, nonpolar amino acid side chains (e.g., phenylalanine, leucine, tryptophan, valine, etc.) are located in the interior of the protein away from the aqueous solvent.[29,31] The hydrophobic effects that promote this distribution are largely responsible for the three-dimensional structure of native proteins. On the contrary, ionized side chains such as lysine, aspartic acid, etc., tend to be on the surface of the molecule to interact with the aqueous solvent.[28,29,31] In addition, the polypeptide chains of larger proteins tend to exist in structural domains independently folded and connected by segments of peptide chains.

The amino and carboxylic groups of amino acids readily ionize.[29] At physiological pH (≈ 7.4), the amino groups are protonated and the carboxylic acid groups are in their conjugate base form. The acidic and basic side chains determine the overall charge of the molecule. The point at which the net charge is zero (i.e., the number of negative and positive charges are equal) is denominated as isoelectric point (pI).[29] When the pH is higher than the protein pI, the protein carries a negative charge, and when the pH is lower, the protein is positively charged. Solubility of proteins varies inversely with how close it is to the pI, being less soluble near the pI.

Taking into account the low stabilities of protein conformations, these molecules are easily susceptible to denaturation,[27] by changing the balance of the weak interactions that maintain the native conformation. Proteins can be denaturated by a variety of conditions and substances such as heating, extreme pH, chaotropic agents, detergents, adsorption to certain surfaces, etc.

14.3.2 Protein Behavior at Surfaces

When a protein is in an aqueous media solution, the molecules fold in the way that their hydrophilic residues are exposed toward the solvent, which leads the protein to adopt a globular structure. The adsorption of globular proteins from aqueous solution to polymeric solid surfaces may generate partial unfolding of the protein conformation, which depends mainly on the primary, secondary, and tertiary structure of the protein and also on the characteristics of the surface.[32] The adsorption of proteins at the liquid–solid interface comprises various steps such as transport of the protein from the bulk solution into the interfacial region, attachment of the protein at the sorbent surface, and the relaxation of the protein on the surface (optimization of the protein–surface interaction).[33] Protein adsorption, which was discussed in detail in Chapter 22 by Alves and Reis, is a result of several processes including[30] electrostatic interactions between the charged protein molecule and the charged sorbent surface; steric interaction due to highly hydrated, flexible oligomeric or polymeric components at the sorbent surface into the surrounding aqueous solution; deposition of a protein layer on the surface of the hydrated polymers and a decrease in their conformational entropy; changes in the state of hydration; and rearrangements in the protein structure. When a protein reaches the sorbent surface, at one side of the molecule the aqueous environment is replaced by the sorbent material. Nonpolar regions of the protein that are located in the interior of the molecule can become exposed to the sorbent surface, where they are still protected from contact with water. As a result, intramolecular hydrophobic interactions become less important as a stabilizing force. Since it is the hydrophobic interactions between amino acid side groups in the interior of the protein that maintain the α-helices structures, a decrease in those interactions may destabilize the helices. Thus, whether adsorption on a hydrophobic surface causes an increased or a decreased ordering in the protein structure depends on the delicate balance between the energetically favorable hydrogen bonds and the conformational entropy of the protein. Normally, the extent of protein conformational changes is greater on hydrophobic surfaces than on hydrophilic surfaces.[30] It is, therefore, of extreme importance to evaluate the occurrence of conformational rearrangements on the protein structure after adsorption, especially when active proteins are desired in the adsorbed state. Furthermore, depending on the degree of conformational changes, different amino acids will be exposed

at the surface, which will then control the interactions between the adsorbed protein and the surrounding molecules or entities.

14.3.3 Catalytic Activity of Enzymes: The Ability to Transform and Control Local Chemistry

Enzymes are sophisticated catalysts that are distinguished from chemical catalysts by their efficacy, specificity, and sensitivity to control.[29] As proteins, enzymes share the same features as other protein molecules, but they are able to increase the rate of a great variety of reactions by lowering the free energy barrier that separates reactants and products. Enzymes accomplish this ability through various mechanisms that depend on the arrangement of functional groups in the enzyme's active site, the region of the enzyme where the catalysis occurs. The noncovalent forces through which substrates and other molecules bind to enzymes are similar to the forces that dictate the conformation of the native proteins. In general, a substrate-binding site consists of an indentation or cleft on the surface of an enzyme molecule that is complementary in shape to the substrate. Furthermore, the amino acid residues that form the binding site are arranged to interact specifically with the substrate.

Due to the dynamic nature of enzymes, through its catalytic activity, these biomolecules may alter the local environment by changing surface characteristics or by consuming or releasing reactive species, which then may influence other processes.

Taking into account the vast number of reactions taking place in biological systems and that many of those reactions are catalyzed by enzymes, a large number of enzymes are expected to occur in nature. In fact, in the sixth edition of the Enzyme Nomenclature (Nomenclature Committee of International Union of Biochemistry and Molecular Biology, 1992), 3196 different enzyme activities were described.[28] In addition, while some enzymes are very specific (they catalyze only one reaction involving only certain substrates), other enzymes act on quite a broad range of substrates. The great diversity of enzymes available in nature, associated with their unique and novel catalytic activities, presages the development of new technologies where the potential value of enzymes can be exploited.

14.4 BONE PROPERTIES AND MINERALIZATION

According to many authors,[34–40] the term *bone* refers to materials structurally characterized as highly hierarchical and complex and which share the basic building blocks of mineralized collagen fibrils.

For many years, the biological interest of bone was not really considered.[41] Nevertheless, over the last 15 years, macro- and microstructural properties of the skeleton have been investigated within a physiological homeostatic environment, revealing important details on normal bone and pathologic condition bioprocesses.[41,42] The growing interest in bone biology has brought considerable amounts of information in terms of skeleton development and physiology and pathology.[43–46] This significant progress led to more questions, some of which are to be discussed in the present section.

Essentially, bone, as a connective tissue,[47] is responsible for ensuring the internal support of higher vertebrate organisms, and the composition of bone is more complex than the majority of orthopedic engineered composites.[48] Bone comprises a mineral phase intimately associated to an organic phase.[49] The presence of an extracellular mineralized matrix and the interaction of carbonate apatite crystals and collagen confer to bone its mechanical properties by means of scaling a balance between rigidity, strength, and elasticity[49,50] and enables crystal formation.[51]

These tissue active functions, such as the homeostasis of mineral ions or the storing of healing cells, make bone a reactive specific surface of fundamental biological value.[48,50,52] The importance of these cells became more evident with the increasing knowledge on the mineralization of bone. Although primarily a physicochemical phenomenon, that is, while first crystals are formed, the later stage of mineral propagation is regulated by cells that define the progress of mineralization.[50,53–56]

14.4.1 Structure and Development of the Bone

The complete understanding of bone properties demands a detailed analysis that ranged from the macrostructural level to the sub-nanostructure of the bone.[34,57] Over the years, several microanalytical, microstructural, and imaging techniques were applied, such as secondary ion mass spectrometry (SIMS),[58] X-ray diffraction,[52] and microscopic methodologies such as transmission electronic microscopy (TEM)[34] and coupling cryotechniques such as anhydrous freeze-substitution[59] and frozen sectioning,[60–62] among others.

Regarding the macrostructure of bone, terms such as *cortical* (or compact) and *cancellous* (or trabecular) define two different morphologies closely related to the functionality of these structures. Mainly the degree of porosity or density characterizes both types of bone, but a reliable distinction can be achieved only by means of coupling microscopic technologies.[50] The basic units of cortical bone are the Haversian systems (osteons) constituted by cylindrically shaped lamellae that result from the dense packaging of mineral salts and collagen fibrils. Between lamellae, small spaces accommodate the mature bone cells, designated lacunae, and project smaller channels, the canaliculi, ensuring nutrient and waste products exchange. Blood vessels are localized in the central Haversian canal together with the nervous tissue.[47,48] On the other hand, cancellous bone is a more loosely organized structure of irregular sinuous convulsions of lamellae, presenting a porous matrix and characterized by the presence of trabeculae, red bone marrow, and absence of osteons.[47,48] Generally, cancellous bone is younger than cortical bone due to its higher metabolic activity and frequent remodeling. The higher homogeneity of cortical bone may be the reason for the lower turnover observed for this particular structure.[50]

Bone organic phase is approximately constituted by 95% of collagen type I and 5% of noncollagenous proteins and proteoglycans.[50] Collagen constitutes the most concentrated protein in the human body, achieving values of around 25% of total protein amount.[47,54] In bone, collagen has two major functions[54,63]: It supports HA formation after mineralization as entered in extravesicular phase by constituting a substratum for mineral deposition,[63] and on the other hand, due to the presence of peptides specific for cell reception, this protein is able to mediate cell attachment.[54]

Bone, as dynamic biological material, is remodeled and resorbed in a turnover process of osteoblast/osteoclast interactive cycles.[64,65]

Osteoblasts, typical protein-producing cells, secrete collagen type I and other noncollagenous protein constituents of the bone matrix involved in bone formation.[56,66,67] In fact, this is the only characteristic that distinguishes osteoblasts from fibroblasts: the ability to produce mineralized extracellular matrix (ECM).[56] In this sense, osteoblastogenesis[68] is responsible for bone matrix production and regulation mechanisms. These cells are known to suffer programmed cell death during fast bone formation, such as fracture healing, showing the importance of this process in the regulation of normal bone functioning.[69–72] Mineralization proceeds differently, depending on the specific bone location where it is taking place. Independent of the mechanism, the initiation and development of the mineralization results from the layer of unmineralized tissue that is produced under the osteoblast membrane, designated by the term *osteoid*.[53] It is deposited onto the bone surface and periodically surrounds the osteoblast cell, gradually decreasing the ability for matrix production and generating an osteocytes layer.[53] Life continuity of these matrix-remodeled cells is guaranteed by the organization of efficient cell–cell communication mechanisms.[50]

Osteoclast cells, originally described in 1973,[73] are hematopoietic in origin, deriving from monocyte-macrophage lineage, which degrades the mineralized matrix produced during normal bone formation.[55,73–76] Osteoclastic bone resorption characteristically occurs on the bone surfaces during bone remodeling and modeling following osteoblast bone production.[77] Bone homeostasis is dependent on a balanced synchronism between bone formation and bone resorption, which is the reason why several bone pathologies, such as osteoporosis, focus on the regulation of osteoclastogenesis.[78] Active osteoclasts exhibit a characteristic phenotype resulting from two different plasma membrane specializations: a clear zone and a ruffled border.[50] Bone resorption occurs by

means of protease secretion dissolving the matrix and by an acidification process that releases bone mineral into the extracellular space and in the ruffled border side.[50,74]

Describing the coupling between bone formation and bone resorption is a result of macroscopic evidences observed in normal and abnormal bone. On the cellular level, indications of the same association have been found. According to recent findings,[50,74] cascades of events with different hormones and cytokines are initially responsible for osteoblast functional activity. In turn, osteoblastogenesis leads to the production of potent osteoclastogenesis stimulatory or inhibitory signals that develop in endocrine or autocrine pathways.

14.4.2 Bone Mineralization

Skeletal cells have been elucidated as the decisive bioelements in the initiation and propagation of bone mineral. Mineral-facing osteoblast surfaces produce matrix vesicles (MV)[79–81] that are selectively delivered in the matrix to function as initial mineral deposition sites and to control the mineral distribution. MVs are exocyted from osteoblasts, as from chondrocytes and odontoblasts, by a budding process that does not require cell death. These vesicular pieces are limited by an active membrane and contain the essential molecules for the initiation of bone mineralization: enzymes, proteins, and lipids.[79–81]

A biphasic mechanism[82,83] is proposed for the mineralization phenomena based on the information acquired over the years on matrix vesicles. Phase I of mineralization is accomplished by the interaction of calcium-binding molecules and phosphate-metabolizing enzymes, present in the MV membrane or near to it. Calcium will be attracted to the MVs by non-energy-requiring calcium-binding lipids and calcium-binding proteins such as calpactin, calbidin, and type X collagen.[82,83] Ion pumps, normal cell strategies for active transport, were also identified.[84–86] On the other hand, the increase in local phosphate is accomplished by phosphatase enzymes such as alkaline phosphatase (ALP), which reside on the vesicle membrane and depend on the availability of ester phosphate from the surrounding medium. The membrane operates as a protected microenvironment for calcium and phosphate entrapment. First, unstable nuclei of calcium phosphate mineral will form, supporting transformation into hydroxyapatite by means of being earlier converted to octacalcium phosphate (OCP).[53] At this point, crystals penetrate and perforate the membrane and are exposed to the cartilaginous fluids. This bioenvironment is characterized by the circulation of insufficient calcium and phosphate on what concerns mineral formation. Preformed apatite from MVs is already present and acts as a template for crystal proliferation. The entire process will be anticipated to a point where calcium and phosphate levels from circulating fluids are already suitable for mineral propagation. In this second stage, the concept is no longer mineral nuclei formation but mineral growth.[82,83] Calcification is known to be mainly controlled by matrix components able to accelerate or retard mineral propagation. In this way, the mineral templates originally produced in the earlier stage will now proliferate essentially by a so-designated physicochemical process.

Many molecules, such as cell-produced matrix components and soluble factors, have been identified[82,87,88] as modulators of bone development under normal function or abnormal conditions. Table 14.1 summarizes some of the most important or studied proteins, growth factors, and other molecules independently of the stage in which their activity is performed, as reported by different studies.

14.4.3 The Interface Bone Implant

To understand the mechanical properties of bone, it is fundamental to consider the different structures of this highly organized material. Furthermore, the study of bone mechanical properties is only well understood when considering that physical activity plays a major role in the modeling and remodeling of bone tissue. Specifically, mechanical stimulation affects osteoblast adhesion, proliferation, and differentiation.[54] These forces are created by the movement and exterior impacts

TABLE 14.1
Examples of Studies Performed to Analyze the Effect of Several Organic and Inorganic Molecules on Bone Development

Molecules	Molecule Type	Effect	Ref.
Alkaline phosphatase	Polypeptide with enzymatic activity	Promotes crystal formation in matrix vesicles	83
Fibronectin	Protein containing synergistic sequences	Used by osteoblast for cell attachment, mainly through RGD–integrin interaction	89, 90
Gla-protein or osteocalcin	Noncollagenous protein	Calcium-binding protein of the matrix that may inhibit mineralization and be involved in bone resorption. Studies show in opposition its ability to function as promoter of HA nucleation	88, 91
Osteonectin	Glycoprotein	Calcium-binding protein of the matrix that may inhibit mineralization. Also suggested to act as matrix mineralization nucleator.	82, 88
Bone sialoprotein	Protein conjugated with sialic acid	Cell attachment to bone matrix	92
Osteopontin	Acidic sialoprotein	Cell attachment to bone matrix	82, 87
Bone morphogenic proteins (BMP)	Morphogenic cytokine	Stimulate differentiation of mesenchymal cells to osteoblasts. Stimulates matrix production.	93
Fibroblast growth factors (FGF)	Cytokine	Stimulate proliferation of mesenchymal cells, osteoblasts, and chondrocytes	94
Transforming growth factor-β (TGF-β)	Cytokine	Stimulate differentiation of mesenchymal cells to chondrocytes and later on to osteoblasts. Modulates FN and osteopontin synthesis. May be involved in the dynamical bone formation/resorption.	94, 95
Dexamethasone	Corticosteroid	Stimulates ALP secretion; TGF-β decreases its activity	96
Interleukin-1 (IL-1)	Interleukin	Stimulates osteoclastogenesis. Involved in bone resorption.	96
Ascorbic acid or vitamin D	Vitamin	May be involved in the dynamical bone formation/resorption. Main importance as regulator of other molecules' synthesis (ALP, osteonectin, and osteopontin).	97, 98

Data from Robey, P.G., *Endocrinol. Metab. Clin. North Am.*, 18, 859, 1989; Eanes, E., in *Calcification in Biological Systems*, Bonucci, E., Ed., CRC Press, London, 1992; Denhardt, D.T. and Guo, X., *FASEB J.*, 7, 1475, 1993; Linde, A. and Lussi, A., *Connect. Tissue Res.*, 21, 197, 1989; Weiss, R.E. and Reddi, A.H., *J. Cell Biol.*, 88, 630, 1981; Globus, R.K. et al., *J. Cell Sci.*, 111, 1385, 1998; Boskey, A.L. and Timchak, D.M., *Metab. Bone Dis. Rel. Res.*, 5, 81, 1989; Fisher, L.W. et al., *J. Biol. Chem.*, 265, 2347, 1990; Anderson, H.C., *Connect. Tissue Res.*, 24, 3, 1990; Caplan, A. and Boyan, B., in *Bone*, Hall, B., Ed., CRC Press, London, 1994; Mundy, G.R. and Bonewald, L.F., *Ann. N.Y. Acad. Sci.*, 593, 91, 1990; Buckwalter, J.A. et al., *J. Bone Jt. Surg.*, 77A, 1256, 1996; Boyan, B.D. et al., *Endocrinology*, 122, 2851, 1988; Lian, J.B. and Stein, G.S., *Crit. Rev. Oral Biol. Med.*, 3, 269, 1992.

and are a similar dynamic environment that is encountered by a synthetic prosthesis after implantation.[99] Moreover, recent observations have recognized the importance of the prosthesis biomechanics concept as the main determinant of implant failure.

The dynamics of the implant site affects the initial events of osteoblast adhesion, determining later on proliferation, differentiation, and mineralization phenomena.[99,100] In this context, several studies on the effect of mechanical forces on initial cell adhesion are arising as achieved by the application of dynamical conditions.[99,100]

In the last decade, protein adsorption was reported by several authors[101–105] as the initial step following the contact of an artificial surface with blood, being objectivated as an important aspect of the material–cell interaction. Whenever an implant material contacts with the body environment,

protein molecules spontaneously accumulate at the solid–liquid interface. Initially, these proteins came from the blood and surrounding fluids and, in later stages, from the normal cell activity such as ECM. This in turn will alter the surface and in many cases also the properties of the adsorbed molecules.[101,102,105] Recent studies[106,107] have shown the variation of the osteoblastic phenotype onto different surfaces known for differential modulation of fibronectin (FN) conformation. The primary mode of adhesion of cells to the ECM is performed by means of integrins, cell membrane proteins that recognize the well-studied RGD peptides (arginine-glycine-aspartic acid).[107,108] Integrins were already demonstrated to be expressed in bone and in bone cell culture.[109] Furthermore, RGD adhesive sequences, so designated due to their ability to bind a specific ligand,[54] are known to be present in several bone-related proteins[110]: bone sialoprotein, collagen, fibronectin, osteopontin, thrombospondin, and vitronectin. Following the receptor-sequence interaction, an intracellular cascade of multiple signaling events can be activated, regulating subsequent cell migration, proliferation, phenotype, genotype, and thus, cell differentiation.[99,111,112]

The success of an implant material for bone-related applications depends on biological, physicochemical, and mechanical characteristics of the whole polyphasic structure. Furthermore, the surface change over time, resulting from the environment reactivity and the tissue response, defines the long-term implant stability.[113] In other words, the integration of the implant is determined by the performance of the device, which largely takes place in the tissue–implant interface.[114] The nature of the material — its shape, topography, chemistry, surface energy, and mechanical properties — can determine which molecules adsorb and their orientation, thus influencing normal bone cellular development.[54,99,114,115] Also, the surgical procedure, bone quality, and patient variables control the healing yield of the implantation, which is decisive in the device's success (see Figure 14.1). In the search for new bone grafts, several goals are desired to be achieved. Besides the rapid-healing medical situations, implant fixation binding generally by the formation of an interfacial matrix similar to bone is sought.

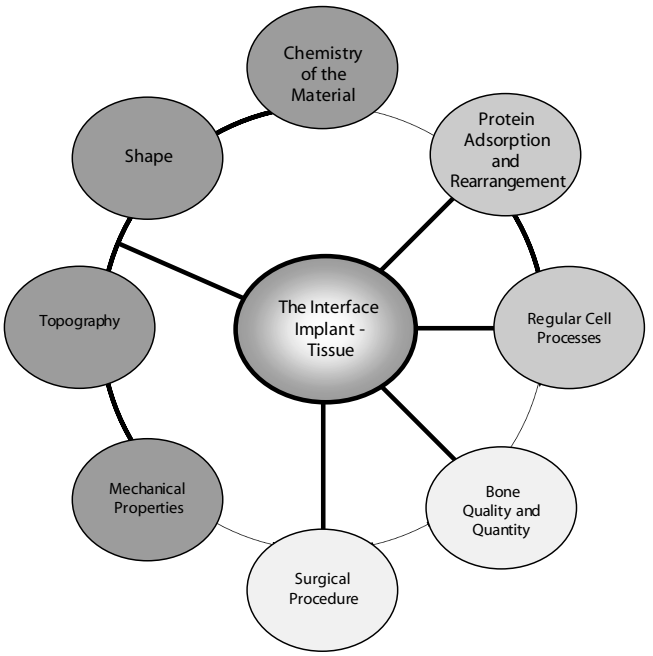

FIGURE 14.1 Factors influencing the implant–bone tissue interface. (Adapted from Anselme, K., *Biomaterials*, 667–681, 2000; Palma, F.D. et al., *Biomaterials*, in press; Puleo, D.A. and Nanci, A., *Biomaterials*, 20, 2311, 1999; Boyan, B.D. et al., *Biomaterials*, 17, 137, 1996.)

The response of the material surface, and to a later extent of the bulk of the material, will undergo significant changes starting from time zero of implantation.[114] Biodegradable materials are susceptible to incorporating ions and compounds from the medium, but also to sending to solution products of the degradation. Material surface can easily alter the phenotypic expression of bone-related cells; namely, molecular weight, polydispersity, wettability, and crystallinity can lead to variations in the normal cell bioprocesses.[116,117] On the other hand, local tissue response starts surface erosion mechanisms originating degradative by-products that can affect the pH of the neighboring surroundings. Furthermore, oxygen tension potentially defines the population that proliferates.[118] The entire environment that is settled around the implanted device will affect the overall cell response by selecting populations, thus delineating the later tissue response.[117,119]

Bone mineral-like structures attract osteoblasts, which after adhesion are able to proliferate and differentiate with the production of extracellular matrix. Calcium phosphates found in bioactive ceramics are an adaptive strategy of mimicking the bone composition, thus allowing for the inclusion of materials that in normal conditions are unable to promote mineralization.[120] When contacting blood plasma, a calcium- and phosphate-rich solution,[121] these surfaces are directed through biomineralization.[121,122] In this process, proteins, the most concentrated organic molecules composing blood plasma,[122] have been found to interact positively or inhibit mineralization. Furthermore, the interaction of mineral nuclei and the organic compounds from the surrounding environment and matrix will depend on several characteristics of the matrix itself, such as geometry, polarity, chemistry, and topography.[123] The matrix production resulting from the initial recruitment of osteoprogenitor cells and later proliferation and differentiation of osteoblasts leads to the search for tissue engineering constructs where osteoinductive factors are combined with osteoconductive materials and osteogenic cell populations.[124] The main objective would be to promote bone growth, starting from supplying bone cues at the bone–implant interface.[114,125] Several molecules have been grafted onto the surfaces to study their effect on the generation of artificial extracellular matrices, such as collagen,[126] RGD sequences,[112,127] or other synthetic peptides,[128] and also the use of osteogenic growth factors such as BMP[129] or TGF-β.[130]

14.5 BIODEGRADABLES AND BIOMIMETIC COATINGS

14.5.1 Biodegradable Polymers

In the last few years, starch-based polymers have been proposed[131–135] as alternative biomaterials for several orthopedic applications. These materials combine a degradable behavior with an interesting combination of mechanical properties.[131–135] Additionally, it has been shown[136–138] that these materials can comply with the biocompatibility requirements of a biomaterial, as defined in international standards, which is not typical of biodegradable systems.

Devices obtained from biodegradable materials present great advantages and are the best alternative for the temporary internal fixation of many different kinds of tissue damage for several reasons.[139–141] It is widely reported[140–142] that during the early stages of tissue healing (bone, tendon, muscle, skin, etc.), the biodegradable implant is capable of holding the healing tissue in place. With the passage of time, the implant decomposes gradually and the stresses are transferred gradually to the healing tissue, avoiding the traditional stress-shielding effects associated with the use of very stiff materials. The other reason is the fact that biodegradable surgical devices do not require a removal operation, which is of substantial benefit, both economically and to the human being involved. In addition, there is the absence of corrosion and fatigue failure and release of metal ions, such as nickel or chromium, which may cause loosening of the implant, patient discomfort, and allergic reactions.[139,140]

Such types of materials, which degrade slowly and predictably in the human body, are useful in several biomedical applications, especially those serving temporary functions such as sutures, bone fixation devices, applications related to reconstructive surgery, scaffolding for cells that re-

create damaged or diseased organs, and controlled-release drug delivery devices.[140,141,143–145] Materials used in such applications include synthetic and natural polymers, ceramics, and ceramic-based composites.

14.5.2 CaP Coating Techniques

As described before, the bioactive materials containing hydroxyapatite or its components, such as calcium oxide (CaO) and phosphate (P_2O_5), are often designated as osteointegrating or osteoconductive materials.[7] Due to the properties of all bioactive glasses, glass ceramics, and calcium phosphate ceramics, such as bioactivity, biocompatibility, and osteogenicity,[18] their applications are found in a variety of fields. Furthermore, they have the capacity to form a mechanically strong interfacial bond with bone.[3] The strength of the bond is generally equivalent to, or greater than, the strength of the host bone, depending on test conditions.[4] Although the mechanical properties of bioceramics are typically not adequate to their proposed function, i.e., they have a flexural strength and fracture toughness that is less than bone and an elastic modulus that is much greater than bone,[145] they still find many applications. In fact, their excellent bioactivity has led to the development of alveolar ridge augmentation, maxillofacial and spinal surgery, otolaryngology, scaffolds for bone growth, and powders in total hip and knee surgery,[9,17,18] where the mechanical properties are not so important.

In the case of using bioceramics in load-bearing clinical applications, the mechanical behavior is very important. One approach to suppress the drawback of the low mechanical properties of these materials has been the use of bioactive coatings applied to a substrate responsible for the mechanical strength.

As a result, in the last few years, calcium phosphates (CaP) coatings were applied to dental and orthopedic implants by different techniques, such as plasma spraying,[9,146] laser ablation,[147] electrophoretic deposition,[148] sol-gel deposition,[149,150] radio-frequency (RF) magnetron sputtering,[151] and electroless deposition.[152] However, most of the available methods for producing adequate CaP coatings are difficult to control regarding the calcium phosphate layer composition, degree of crystallinity, and capability to generate strong bonds with the substrates, i.e., to produce coatings with a good adhesion.[153–155]

For example, the problems associated with electrophoretic deposition are the poor adhesion between the coating and the substrate and the formation of other phases.[155,156] Plasma spraying is the most common commercial technique and is approved by the Food and Drug Administration (FDA) for applying CaP coatings to implant surfaces. This technique has several disadvantages, however, including the formation of other phases, such as tricalcium phosphate or calcium oxide, the poor adhesion to the substrate, the fact that it is not possible to coat on porous implants, and its restricted line-of-sight application.[155–156] Besides that, the crystallinity of plasma-sprayed coatings is not uniform, and the coatings consist of a mixture of crystalline and amorphous regions.[157] If the CaP material is released from these heterogeneous coatings, the resultant particles may initiate inflammation in surrounding tissues.[157] It has been claimed[158] that to deliver better *in vivo* stability for long-term performance, the HA coatings should be highly crystalline, thus achieving a lower degradation rate compared with amorphous or partly amorphous coatings. Therefore, a coating is expected to possess long-term stability and at the same time act as a reservoir of calcium and phosphate ions for inducing greater bone formation and bone bonding.[159]

However, others types of coating methodologies have been used to induce CaP deposition. For instance, Kim et al.[160] have demonstrated that heterogeneous nucleation and growth of bonelike apatite layer can be induced by alkali-treated metal in body environment, i.e., hydroxylation of metal oxide surfaces placed in simulated body fluid (SBF) for different periods of time. Unfortunately, these techniques require heat treatments under high temperatures of at least 500ºC, which result in the degradation of HA into various calcium phosphate phases, and its composition tends to differ from bone apatite in chemical composition and in crystal structure, such as the number

of defects of apatite crystals or the size of the crystallites.[14] Also, the high temperatures involved make them unsuitable for coating organic polymers.

In order to overcome the aforementioned disadvantages and utilize a wider range of substrates than the traditionally used metals, it is desirable to employ a coating technique that does not require high processing temperatures. This would open up the possibility of using nonbioactive materials involving metals, ceramics, and organic polymers that have the necessary strength and toughness to act as implant materials on load-bearing sites.

14.5.3 Biomimetic Coatings

Nature has been used as a source of inspiration to design practical materials and systems. The concept of biomimetics has been explored by several authors[14–16,161] under different perspectives. Based on this concept, Kokubo et al.[14] developed a technique of coating different organic materials as well as inorganic and metallic materials with bioactive layers, which is designated as biomimetic coating.

The main aim of this biomimetic process is to mimic the biomineralization, leading to the formation on the surface of the substrate of a bonelike carbonated apatite layer. The methodology has been claimed to be very useful for producing highly bioactive and biocompatible composites with different mechanical properties.[162]

The crystal size of a biomimetic coating is smaller and the crystallinity is more comparable to bone mineral than to large and sintered hydroxyapatite particles produced by plasma spraying.[157] Therefore, these bioactive layers have the capacity to develop an interfacial mineralization much more rapidly than HA or TCP implants,[6] and such bonelike apatite is supposed to provide a more preferential environment for bone cell seeding and proliferation than sintered HA.[163]

The substrates (such as ceramics, metals, and polymers) coated with the bonelike apatite by this process have great potential as bone-repairing materials, since they can exhibit not only high bioactivity, but also mechanical properties analogous to the natural cancellous bone.[164]

The original biomimetic method for coating apatite in different substrates includes two steps as described in the following sentences.[14,162] In order to form apatite nuclei on the substrates of different materials, the substrates are placed typically near $CaO\text{-}SiO_2$-based glass (MgO 4.6, CaO 44.7, SiO_2 34.0, P_2O_5 16.2, CaF_2 0.5 wt%) particles immersed in a SBF[165] with ion concentrations (Na^+ 142.0, K^+ 5.0, Mg^{2+} 1.5, Ca^{2+} 2.5, Cl^- 147.8, HCO_3^- 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5 m*M*) nearly equal to those of human plasma at 36.5ºC (first treatment designated as nucleation period). It was also confirmed that not only $CaO\text{-}SiO_2$-based glasses but also the binary $Na_2O\text{-}SiO_2$ glasses are effective as catalysts for the apatite nucleation.[166] The glass particles release large amounts of calcium and silicate ions. The silicate ions are absorbed onto the surface of the substrate to induce the apatite nucleation, and the calcium ions increase the degree of supersaturation with respect to apatite in the SBF, which accelerates apatite nucleation. Then, in order to induce the apatite nuclei formed in the first treatment to grow on the substrate *in situ* to form an apatite layer, the substrate is immersed in another solution, e.g., 1.5 SBF with ion concentrations (Na^+ 213.0, K^+ 7.5, Mg^{2+} 2.3, Ca^{2+} 3.8, Cl^- 223.2, HCO_3^- 6.3, HPO_4^{2-} 1.5, SO_4^{2-} 0.8 m*M*) 1.5 times those of the SBF at 36.5ºC (second treatment designated as growth period). The thickness of this apatite layer increases with the increment of the immersion time in the second treatment, and the growth rate of the apatite layer increases with the increment of the ion concentrations of the second solution.[162]

By this method, apatite layer has been successfully deposited on ceramics, metals, and polymers, such as alumina glass,[14] zirconia ceramic,[14] titanium (Ti),[14] polyethylene (PE),[153,167] poly(methyl methacrylate),[14,167,168] ethylene–vinyl alcohol copolymer, polyethylene terephthalete (PET),[167–169] polyether sulfone (PESF),[153,167,168] polyamide 6 (Nylon 6),[153,167] and poly(vinyl alcohol) (PVA).[167] One aspect that is important in biomedical applications with this type of coating is to obtain a strong adhesion of the apatite layer to the substrate. For example, in the case of organic substrates, the adhesive strength has been improved by increasing the surface roughness of the substrates or

by increasing the amount of polar groups on the substrate surface using a variety of surface modification methods.[153,170–172]

This biomimetic coating method has the following advantages over conventional ones such as plasma spraying[173]: (1) Uniform coatings can be reproduced onto complex-shaped or microporous implants due to the fact that this method is a non-line-of-sight coating process; (2) the biomimetic CaP layer is expected to show higher bone-bonding ability; (3) it is a simple and cost-effective way to produce CaP coatings; and (4) there is no adverse effect of heat on substrates due to the low processing temperature. Furthermore, this coating method has three important characteristics, namely[174] (1) the control of solution conditions, including ionic concentrations (supersaturation levels), pH, and temperature; (2) the use of functionalized interfaces to promote mineralization at the substrate surface; and (3) the formation of dense calcium phosphate films without the requirement of subsequent thermal treatments.

The functionalized surfaces are believed to be analogous to nucleation proteins in biological systems in their ability to provide energetically favorable interfaces for heterogeneous nucleation and growth of inorganic films from supersaturated solutions.[174] However, it is important to stress herein that, even with all these advantages, these biomimetic coating methodologies continue to have some problems with depositing an apatite layer on materials with complex shapes, due to the fact that in the first treatment step, the apatite nuclei are formed only on the material surface that is directly facing the glass particles.[175] To solve this, Kokubo et al.[175] reported that in the first treatment step, instead of using glass particles, the material could be immersed in a sodium silicate solution, which is regarded as a solution of the Na_2O-SiO_2 glass having catalytic effect for the apatite nucleation. As mentioned previously, it is expected that silicate ions, which can induce the apatite nucleation, are attached to the surfaces of the substrates, and then the apatite is formed on them by the subsequent soaking in 1.5 SBF.

In contrast, Reis et al.[176,177] report that the apatite coating is deposited successfully not only on starch-based blends and bioinert polymers such as polyethylene (HMWPE), but also on materials with complex shapes such as polyurethane foams (PU) by using a similar methodology to the original biomimetic method. Furthermore, this methodology allows for the coating of not only the surface, but also the bulk of open cell foams.[176,177] The difference in this process is the fact that the entire surface of the polymer or other material is covered with the glass particles (a slurry prepared in water), which is able to form the apatite-like layer. The production of such biomimetic films on biodegradable polymers, prior to implantation, might allow for the development of bone-bonding, bioresorbable implants and fixation devices, bone replacement materials, and tissue engineering scaffolds.[178]

However, for all coatings, there is the need for careful consideration of the final coating chemistry, always thinking about the future implantable materials, and especially it is needed to understand the role of the dissolution of CaP coatings, which plays a part in this complex bone integration process.[179] The ion release from CaP coatings may indirectly affect cellular processes involved in bone integration through altered ligand–cell receptor affinities, varied calcium and pH-dependent enzyme kinetics, and a compositionally or structurally altered extracellular matrix protein environment.[179,180] The factors that affect ion release from thin-film coatings include CaP chemistry, coating roughness, and extent of coating strain.[179]

14.6 PROTEINS AS NATURE'S CRYSTAL ENGINEERS: HOW PROTEINS MANIPULATE THE MICROSTRUCTURE AND PROPERTIES OF MINERALS

14.6.1 Mineralization Process

The deposition of minerals on a substrate (heterogeneous process) involves four different development stages.[181,182] Individual ionic groups that are destined to form the nucleation core diffuse

randomly through the solution to adsorb on the substrate surface and then form stable clusters. Nucleation involves the formation of nuclei on the surface of a substrate present in the aqueous medium, triggered by electrostatic interactions.[181,183] Nucleation occurs at precise sites, and the substrate seems to orient the nucleus. The involvement of the substrate in the nucleation stage is therefore critical for the control of the nucleation rate as well as for the respective spatial organization. The stable critical nucleus grows by further ionic deposition. Crystal growth and termination are dependent on the level of saturation and occur through surface-controlled processes.[183] The surface of the growing crystal contains active sites of higher binding energy, which drive the further incorporation of ions into the solid phase. The direction of growth seems to be governed by the substrate and the neighbor particles growing in the same substrate. Space restrictions induce these particles to fuse together. The role of the substrate in controlling inorganic nucleation is to lower the activation energy by reducing the interfacial energy.[183,184] By lowering the activation energy through specific molecular interactions at certain positions on the substrate, both the rate of mineral nucleation and the site of inorganic deposition can be highly regulated. The main effects associated with this mechanism are[183] changes in the rate of nucleation, site-specific organization of nucleation sites on the substrate, structural selectivity of mineral polymorphs, and crystallographic alignment of nuclei on the substrate surface.

14.6.2 Crystal Engineering Capability of Proteins

Biomineralization is a highly complex event involving the selective recognition and deposition of calcium ions mediated by proteins, followed by the formation of mineral phase comprising crystallites with specific orientation and morphology.[185] It is well known that acidic (negatively charged) molecules play an important role in biomineralization processes,[184,186] by controlling the nucleation, growth, size, and morphology of mineral phases.[187] The mechanism through which organic macromolecules and organic structures control the synthesis, construction, and organization of an inorganic mineral is, however, not clear. In biological systems, it has been observed that small quantities of certain macromolecules, such as proteins and polysaccharides, are able to change the microstructure of minerals by adsorbing to specific crystal planes.[187] These macromolecules share some common structural features, such as the high content of carboxylic groups (aspartic and glutamic acid residues).[184,185,188,189] Therefore, it is believed that the specific arrangement of acidic groups might allow for the establishment of interactions between the organic chain and the mineral precursor ions (calcium and phosphate ions), leading to local ion saturation and to the growth of crystals in a particular orientation. Figure 14.2 shows how these negatively charged amino acids can interact with calcium ions.

In nature, there are many examples of structures where minerals are complexed with organic molecules to form hybrid materials, including the structure of vertebrates laminar bone,[15,16,188,190,191] dentin,[15,16,190,191] mollusk shells,[15,16,187,191] composite fibrils formed by biogenic silica deposition in plants,[188,191] the membrane of some bacteria,[188] etc. Weiner and colleagues[191] believe that the way by which the mineral phase and the organic material are organized is the key factor in contributing to the distinct mechanical properties of these biological materials. These authors suggested that the different types of organization might constitute a strategy created by the organism to reduce the extent of mechanical anisotropy. In this sense, different molecules have been used to control the nucleation and growth of mineral phases and consequently manipulate their properties (structure, crystallinity, morphology, mechanical strength).[185,188,192,193] The major role of these molecules may be either templating or enzymatic effects.[16] A macromolecular template could provide stereochemistry and physical adsorption for the inorganic formation. On the other hand, an enzyme could regulate inorganic phase synthesis by controlling local chemistry.[16] For instance, it is believed that alkaline phosphatase, the enzyme that catalyzes the hydrolysis of phosphomonoesters with the release of inorganic phosphate, may have such an effect by controlling the kinetics of inorganic phosphate production and therefore their local concentration, which then can complex calcium ions

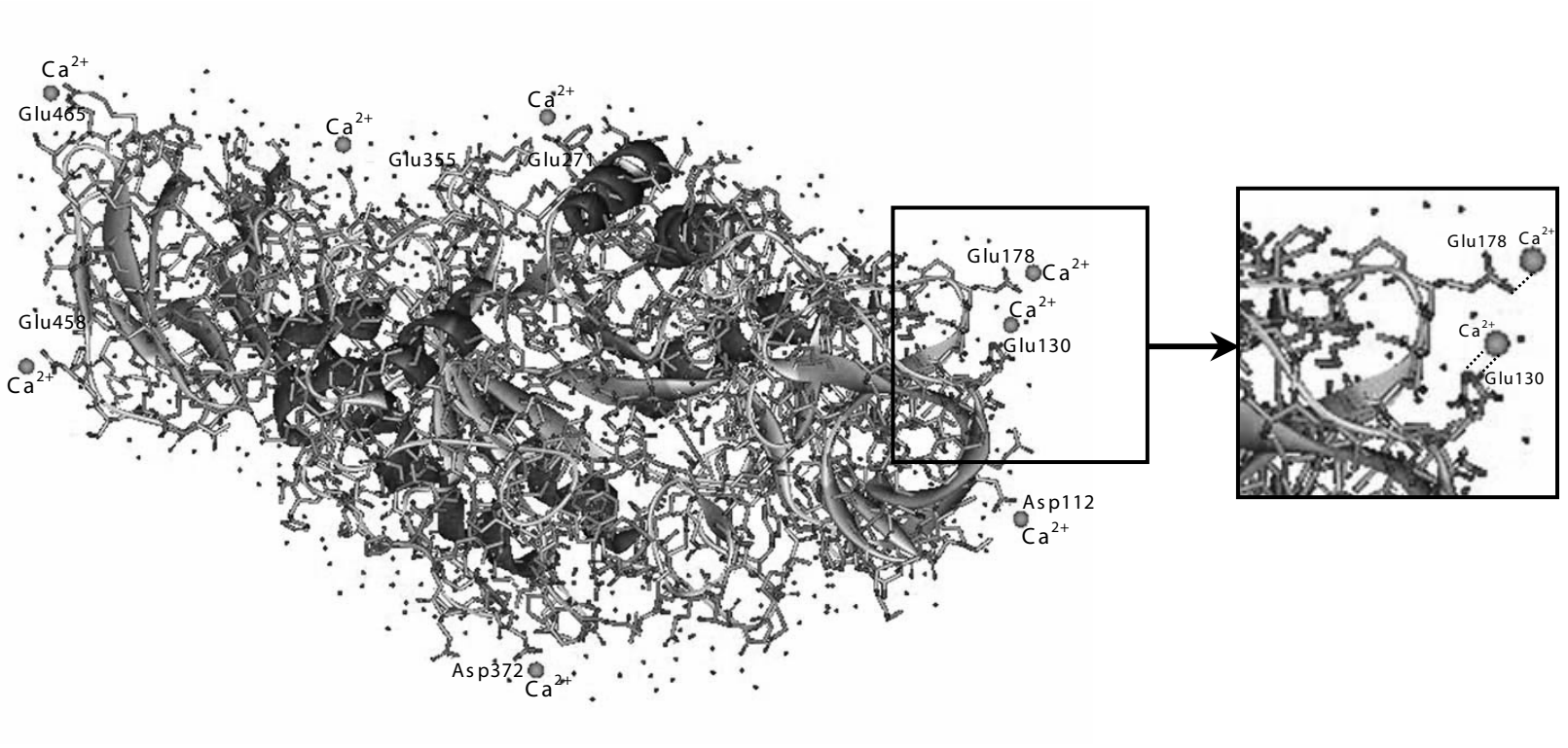

FIGURE 14.2 Ribbon representation of the structure of α-amylase from *B. amyloliquefaciens* showing, in stick representation, the acidic side chains (aspartate and glutamate) exposed on the surface of the molecule. In detail is shown the ionic interactions between the negatively charged amino acids and Ca^{2+} ions.

originating calcium phosphate deposits.[194] On the other hand, the entrapment of biomacromolecules in mineral phases may contribute to strength materials[188,195] and also for stabilizing the mineral contents.[195,196] Supramolecular structures can also be used as templates for the controlled nucleation and growth of inorganic materials, provided that the organic surfaces contain functional groups that can interact with ions present in supersaturated solutions. For instance, Aizenberg and coworkers[193] patterned a metal substrate with self-assembled monolayers to control the crystal nucleation. The system consisted of a monolayer possessing different nucleating activities (an array of acid-terminated regions separated by methyl-terminated regions). After immersion in a calcium chloride solution and exposure to carbon dioxide, they obtained an ordered crystallization of calcite in the polar regions, where the nucleation rate is faster. Crystallization is restricted to acidic-terminated regions and does not occur on the methyl-terminated regions. The nucleation density may be controlled by varying the area and the distribution of the polar regions. It is possible, therefore, to obtain different crystallographic orientations and distributions by selecting other substrates and functional groups. Such achievement is extremely useful for the development of advanced inorganic materials, such as ceramics or coatings.

Differences in the amino acid composition, and in particular the number and orientation of acidic groups exposed at the protein surface (protein conformation),[181] affect the mineral formation in distinct ways. For example, some proteins are known to act as nucleators, such as bone sialoprotein and dentine phosphoryn, whereas osteocalcin and osteopontin have an inhibitory effect.[197] The incorporation of proteins in crystals normally leads to changes in the number, size, and distribution of crystal aggregates, but the extent of these effects depends on some parameters such as the protein concentration[181,198] and the way these biomacromolecules are present in solution (free or immobilized).[181,189] As the protein concentration increases, there is a larger surface area and an increased number of functional groups to interact with the growing crystal nuclei, which facilitates crystallite aggregation. At certain concentrations, however, proteins may have an inhibitory effect.[198] It was also reported that, when present in solution, some proteins were capable of inhibiting the formation of calcium phosphates, either by binding calcium or phosphate[199] or by adsorbing onto apatite surfaces,[199] thus blocking active growth sites. The immobilization of those proteins (adsorbed as a film or bound to a support material) was shown, on the contrary, to initiate mineral formation.[200,201] It is believed that, when the protein is adsorbed, it will complex with calcium at the surface, inducing precipitation, whereas the complexation in solution retards the precipitation. Campbell and Nancollas[199] investigated the ability of various salivary proteins to mineralize hydroxyapatite, when immobilized as films, and found that salivary amylase was shown to be the most active protein in inducing the nucleation and growth of calcium phosphate.

An important consequence of growth inhibition in the presence of certain additives is that the crystal morphology can be dramatically changed. Low- and high-molecular-weight additives, including proteins extracted from biominerals, can induce modifications on the crystal morphology by changing the relative growth rates of different crystal faces through molecular-specific interactions with particular surfaces.[183,187] Electrostatic, stereochemical and structural matching are important factors that significantly modify the surface energy or mechanism of growth, or both. Fast growth along one axis alone gives rise to a needle-shaped crystal, whereas fast growth along two directions produces a plate-like morphology and equal rates of growth in all directions yield isotropic habit (crystal morphology) such as a cube or an octahedron.[183] For example, it has been observed that the presence of monosaccharides, such as glucose, induced the precipitation of needle-shaped hydroxyapatite crystals elongated along the *c*-axis.[190]

14.7 PROTEIN INCORPORATION ONTO BIOMIMETIC COATINGS

The biointegration of biomaterials implicates a series of cellular and extracellular matrix events, some of which take place at the tissue–implant interface and which, in part, reflect the host response to the bulk and surface characteristics of the implanted material.[114] Furthermore, due to the com-

plexities of *in vivo* environment, the science of the bone–implant interface is still not fully understood, in particular the role played by different biomolecules, and their influence on initial bioadhesion, mineralization, and coating dissolution has not received great attention.[198,202] It is well known that protein adsorption constitutes one of the earliest events at the biomaterial–tissue interface that strongly influences the subsequent interactions of many different types of cells with the surfaces and also determines the initial cellular response to the adsorbed surfaces.[203,204] As proteins from the biological fluids come in contact with synthetic surfaces, it has been hypothesized[202,203] that cellular adhesion, differentiation, and the production of extracellular matrix will be affected. Today, it is known that the proteins do more than facilitate mineralization. They organize the extracellular matrix, control cell–cell and cell–matrix interactions, and provide signals to the bone cells.[205] Also, there are many additional enzymes, matrix proteins, and, of course, growth factors that contribute to the formation of bone and can induce a specific cell and tissue response — in other words, to control the tissue–implant interface with molecules delivered directly to the interface.[114,205] Studying their distribution, modification, and *in vitro* effects remains essential. Additionally, the manner in which the mineral is deposited, the orientation of crystals, and their size is influenced by the proteins.[206] All the factors contribute to the strength of the mineralized tissue and stabilize the mineral contents. *In vivo*, the proteins play an important role in modifying and determining the physical and chemical properties of the tissue. In the meantime, adsorbed proteins also modulate cellular interactions that play an important role in hard-tissue regeneration.[196]

Only a few works can be found in the literature concerning the investigation of the influence of incorporation of proteins and active enzymes on the formation of CaP coatings produced by means of using biomimetic routes.[198,207–211] Therefore, this possibility remains greatly unexplored and can constitute a novel approach to produce coatings with tailorable properties, which simultaneously exhibit controlled biomolecule release and bioactive behavior. This approach is attractive because it can be used to control the release of biomolecules (at controlled rates) as a function of specific cell and tissue responses over time.[114] Also, the CaP coatings have high affinity for proteins, which allows for easier binding and makes them ideal carriers for bioactive peptides and bone growth factors (bone morphogenetic proteins [BMPs]) that allow regeneration of hard tissues.[212]

De Groot et al.[195,213,214] demonstrated that the bovine serum albumin can be successfully incorporated into the crystal latticework of mineral matrices coating metal implants when these are prepared by the biomimetic coprecipitation of the relevant components. In addition, due to the degradation of these biomimetic coatings, the protein molecules are released gradually from these coatings[195] rather than in a single rapid burst, as is the case with superficially adsorbed ones, rendering biomimetically prepared coatings of value as slow drug-release systems.[214]

This biomimetic coprecipitation is based on wet-chemistry technique, i.e., acid etching, boiling diluted alkali incubation, precalcification, and immersion in a supersaturated calcification solution.[215,216] This technique produces CaP coatings at physiological temperature (37ºC), which has an important advantage over the conventional coating technique of plasma spraying: the possibility of making a protein delivery system by coprecipitation of osteogenic proteins in the coating with the expectancy of preserving their biological activities.[213]

For example, it has been demonstrated that a titanium mesh coated with CaP coating loaded with BMPs can induce ectopic bone formation and also, due to the CaP coating, can exhibit an osteoinductive behavior.[217]

Our research group has also been studying the effect of the incorporation of protein molecules, namely bovine serum albumin (BSA) and α-amylase, on the properties of biomimetic calcium phosphate coatings.[218] The reason for choosing the traditional biomimetic coatings is the fact that they are normally produced in a simulated body fluid with similar chemical composition to that of human blood plasma under physiological conditions of temperature and pH. In addition, the incorporation of proteins in the coatings under physiological conditions may be suitable for preserving their bioactivity and consequently for producing coatings with carrier potential for the delivery of different biomolecules. For instance, with the plasma-spraying technique, no active

biomolecules can be added during the preparation of CaP layers, due to the extremely high temperatures generated by this coating technique.

In these studies,[218] biodegradable blends of corn starch with synthetic polymers were used as substrate, and bioactive glass (45S5 Bioglass®) was used as nucleating agent to produce bioactive coatings through an adapted biomimetic technique (as described in Section 14.5.3 of this chapter). Bovine serum albumin (BSA) was used as a model protein in order to simulate more closely the conditions found *in vivo*, since the albumin is one of the proteins that first contact an implanted foreign body. Additionally, it is present in human serum and in plasma at a concentration of 38–50 g/l,[219] and consequently, this protein not only has the highest probability of contact due to its concentration, but also is undoubtedly the first that can surround foreign body with the released blood.[219] α-Amylase, a starch-degrading enzyme, was used with the aim of tailoring the degradation rate of the starch-based biomaterial.

Taking into consideration the variety of side chains present on the protein molecule, different types of interactions can be established between proteins and other organic and inorganic molecules. Another aspect to be considered is related to the net charge of a protein, which depends on its isoelectric point (pI) and the medium pH. In this way, depending on the medium pH, different electrostatic interactions can be formed between the proteins and the various ions present in the simulated fluid solutions, thus altering the formation of the CaP coating. At the physiological pH (7.4), BSA and α-amylase are negatively charged and may therefore strongly adsorb Ca^{2+} ions. Serum albumin and most α-amylases contain calcium-binding sites,[220,221] and the presence of these ions may affect the conformational stability and function of these proteins and consequently their adsorption behavior. The binding of Ca^{2+} ions by these proteins would lead, therefore, to a depletion of Ca^{2+} ions available for the formation of CaP surface layer.[211]

On the other hand, the interaction between the protein/enzyme with the starch polymeric material (substrate) needs to be considered. The dominating interactions involved in protein adsorption to uncharged carbohydrate polymers, such as starch, are related to hydrogen bonding, hydrophobic and van der Waals type.[222] The adsorption of polysaccharide-degrading enzymes to insoluble polymeric substrates is a prerequisite step before catalysis, and these enzymes exhibit high affinity and bind specifically to their substrates. α-Amylase is an endo-specific enzyme that catalyzes the hydrolysis of $\alpha(1\rightarrow4)$ glycosidic linkages of starch to maltose and dextrins, reducing the molecular size of starch. The production of reducing sugars during the various stages of incubation[218] revealed that the α-amylase, incorporated within the nucleating agent or in the immersion solution, had adsorbed on the material surface and hydrolyzed the starch substrate. The adsorption of the enzyme on the material and the release of degradation products may also have an effect on the formation of CaP layers.

Taking into account the multiple interactions that can be established between the proteins, the substrate, and the various ions present in solution during the nucleation and growth stages makes the biomineralization process in the presence of biological molecules a rather complex subject. The preadsorption of proteins/enzymes on the biomaterial surface changes the surface conditions and thereby the formation of the CaP film morphology, structure, and composition.[209] During the nucleation stage, a protein can adsorb on the substrate, which can lead to the formation of CaP films with different morphology and thickness. As it can be observed in Figure 14.3, after 3 days of immersion in 1.5×SBF during the growth stage, the entire surface of SCA (blend [50/50 wt%] of corn starch with cellulose acetate) substrate was covered with a CaP film. However, it can be seen that the addition of biomolecules in the nucleation stage led to the formation of a film more dense and compact than the one obtained in the control. As it was mentioned before, serum albumin is the major calcium-binding protein present in the blood, and it has up to 19 calcium binding sites on its imidazole groups.[220] The pI of BSA is 4.7,[208] and it is accepted that at higher pH values, albumin undergoes a neutral-to-acidic transition and becomes negatively charged.[220] In this study, where pH 7.40 was used, albumin molecules can bind Ca^{2+} ions to its electrostatic sites. Furthermore, these cations can act as bridges between the negatively charged proteins and the hydroxyl groups preexposed on the SCA surface. These results indicate that the substrate and surrounding fluids

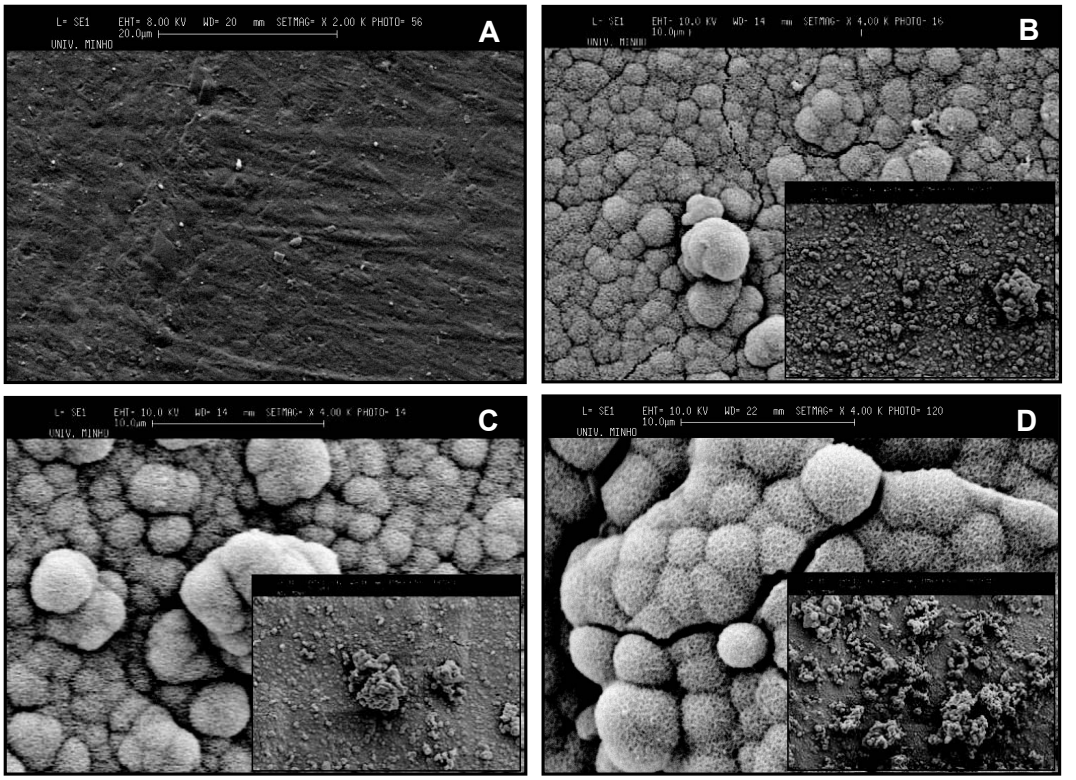

FIGURE 14.3 SEM micrographs of the CaP coatings on the surfaces of SCA before coating (A) and after 3 days in SBF (growth stage). (B) Control, (C) BSA, and (D) α-amylase added in the nucleation stage.

play an important role in determining which protein will adsorb preferentially onto the surface. Thus, it may be assumed that the BSA coated the substrate, and since it is negatively charged at physiological pH, this layer could provide favorable sites for adsorption of Ca^{2+} ions.[220]

Further information on this type of study may be found in Reference 218. However, due to the complexity of the system and to the number of variables involved, further studies need to be carried out. In any case, it is important to state herein that the results of our work showed that through a biomimetic calcium phosphate coating technique, it is possible to incorporate proteins and active enzymes able to hydrolyze a biodegradable starch polymeric material and consequently to control its degradation rate. This work opens, therefore, new perspectives to incorporate other bioactive agents, such as growth factors or specific enzymes, using this biomimetic method in order to induce a cellular response or other desired effect. Several works are presently ongoing within our group concerning this type of research.

14.8 APPLICATIONS AND NEW PERSPECTIVES

In spite of a continuous growth in the general quality of life, as well as an increase in the lifetime of a large number of patients, there has been a growing interest and significant progress in the research and development of biomaterials over the last two decades. However, it is essential to remember that no man-made spare part will be as good as the living part it replaces. Implants cannot repair themselves like most living tissues, which have the capacity to regenerate and repair themselves.[223,224] No implant has biomechanical properties equivalent to the tissue it replaces.[223,224] Therefore, every implant is a compromise, a balance between biochemical compatibility and biomechanical compatibility; if either is wrong, an implant will become loose and fail.

There are a large number of technologies (electronics, biotechnology, biosensors, materials fabrication such as ceramics, nanocomposites) that are using proteins, enzymes, peptides, and functional groups to act as nucleators, anchoring units, or growth modifiers for the synthesis of inorganic materials.[15] This approach may also be very useful in many other applications, such as the development of novel materials for biomedical applications. Biomolecules, with different affinities for a certain polymeric material, may be anchored on its surface with favorable orientation in order to control the nucleating sites, growth kinetics, and final structure of inorganic crystals. This may be achieved by molecular design of recombinant proteins via genetic engineering proteins or by site-directed mutagenesis of existing proteins to obtain peptides or proteins that will bind specifically and selectively to inorganic surfaces. On the other hand, these biomolecules may also have biological effects (cell adhesion, proliferation, and differentiation) that may be useful in tissue engineering applications for controlling tissue response.

Design strategies for creating a biomimetic coating that has a dual beneficial effect — on one side its osteoconductive properties and on the other side its ability to act as a drug carrier delivering therapeutic agents (proteins and growth factors) directly to the interface — might have a very promising future. In fact, we believe that these coatings are quite promising as an approach to stimulate bone growth, combat infection, or achieve other desired effects.

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Part III

Systems for Controlled Release of Bioactive Agents

15 Strategies for Delivering Bone and Cartilage Regenerating Factors

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CONTENTS

15.1 INTRODUCTION TO DRUG DELIVERY

Drug delivery systems are not a new subject in the pharmaceutical area, mainly due to the high interest by the pharmaceutical companies in ways to improve patient compliance concerning drug taking.[1] The first drug delivery system was created back in the 1940s, in order to raise drug levels in blood. In the 1970s, these systems were being developed in a commercial way, but they were very rudimentary.[2,3]

Drug delivery routes are normally[3,4] (1) oral, for pills and syrups; (2) rectal; (3) intravenous, for intravenous solutions; and (4) topic, as for eyedrops. These conventional systems of drug delivery have a major disadvantage in that the concentration of drug decreases to a minimum after a period of time, leading to the need for another dose of drug in a short time interval. Another problem is that the drug will be distributed systemically, throughout the body of the patient.[4,5] The development of medication that allows for the reduction of the frequency of the dosage, thus amplifying the action range of the drug, seemed a very unworthy task; it was also observed that chemically attaching a drug to a polymer (drug macromolecule conjugate) may alter such properties as its distribution in the body, rate of appearance in certain tissues, solubility, or antigenicity.[4,6] These two reasons led to the development of drug delivery systems, whose aim is to facilitate the dosage and duration of the drug effect, causing minimal harm to the patient and improving human health,[1,4] since they allow for the reduction of the dosage frequency[7] and are nontoxic.[6]

Biodegradable delivery systems generally release drugs by the following mechanisms[4,8]: diffusion, chemical reaction, or solvent activation. The release of a drug from a matrix is primarily controlled by diffusion of the drug through the polymer and the dissolution rate of the drug, with the erosion of the polymer being an additional important factor.[9] With regards to biodegradable materials, as soon as the drug-containing system comes into contact with the external liquid environment, it enters the matrix, resulting in a swelling process, which allows for the diffusion of the drug into the external environment.[9] For biodegradable polymers, it is essential to recognize that degradation is a chemical process, whereas erosion is a physical phenomenon dependent on dissolution and diffusion process. Factors influencing release rate include drug molecular size and loading into the polymer, polymer composition and molecular weight, and the dimensions and shape of the matrix.[4,7,10,11]

For controlled delivery systems, bulk properties that need to be considered include molecular weight, adhesion, solubility based on the release mechanism (diffusion[4,11–13] or dissolution-controlled), and its site of action.[7,10,12–14] Structural properties of the matrix, its micromorphology, and pore size are important with respect to mass transport (of water) into and (of drug) out of the polymer.[7] A problem common to many systems is that they display either a constant release rate or the release is dependent on degradation of the system with time[15] and the degradation suffered in the acidic environment of the stomach.[6] Of great importance is the assurance that the delivery system biological activity is preserved throughout manufacturing, storage, delivery, and release[16]; otherwise its use would be worthless.

Drug delivery systems applied to bone and cartilage tissue engineering are finding increasing interest due their potential to enhance bone and cartilage repair and regeneration. Ideally, the combination of bioactive drug delivery systems, in order to promote bone bonding, with specific growth factors that would be released at a desired rate over time, thus promoting the repair/regeneration phenomena, seems the ideal combination of features to be held by such a delivery system, as will be the focus later in this chapter.

15.2 OUTLINE OF THERAPIES AVAILABLE IN THE DRUG DELIVERY FIELD

Drug delivery systems can take the form of microspheres,[13,17,18] nanospheres,[19–22] hydrogels,[23–25] capsules,[26–28] transdermal membranes,[23,29] and liposomes.[4,7,30–33] Their use ranges from the release of growth hormones, anti-inflammatory agents, anticancer agents, and antibiotics, to gene therapy, diabetes, delivery of contraceptives, and respiratory sickness such as asthma,[1,4,34–37] as well as many others. In this chapter, we will focus mainly on injectable or implantable drug delivery systems, such as micro- and nanoparticles and scaffolds that can present a dual function: to support tissue regeneration and to enhance it by itself or by loaded therapeutic agents. The tissue engineering applications of scaffolds as carriers for delivering bone and cartilage active agents will be covered later in this chapter.

15.2.1 Microparticles/Spheres

Polymer microspheres have attracted attention as carrier matrices in a wide variety of medical and biological applications, such as affinity chromatography, immobilization technologies, drug delivery systems, nuclear imaging, and cell culturing. Various parameters including particle size and size distribution, porosity and pore structure, and surface area are considered to describe the overall performance of polymer microspheres in these applications.[38] Polymer spheres with sizes up to 2 mm are produced by various processes, including precipitation, spray drying, and suspension, emulsion, and dispersion polymerizations.[38,39] If the drug delivery systems are fabricated as microspheres, they can be injected with a syringe[40,41] or administrated intranasally as a dry powder,[41] thereby avoiding surgical implant. In the case of bone and cartilage, they can be injected or be combined with the scaffold used for implantation on the repair site.

15.2.2 Nanoparticles/Spheres

The development of intravenously administrated carriers with blood circulation times long enough to continuously deliver drugs (e.g., anti-inflammatory) and other bioactive compounds, imaging agents, or other entities to specific sites of action[6,42–44] has been a major challenge. The desired features of such a carrier include[36,43] (1) that the agent to be encapsulated comprises a reasonably high weight fraction (loading) of the total carrier system (for example, more than 30%); (2) that the amount of agent used in the first step of the encapsulation process is incorporated into the final carrier (entrapment efficiency) at a reasonably high level (for example, more than 80%); (3) the ability to be freeze-dried and reconstituted in solution without aggregation; (4) biodegradability; (5) small size (between 10 to 5 μm); and (6) characteristics to prevent rapid clearance of the particles from the bloodstream.

Nanoparticles offer specific advantages over liposomes, because they increase the stability of drugs/proteins and possess useful controlled-release properties,[36] which is of major concern when they are released in physiological fluids (e.g., blood) or organs (e.g., lungs), because these particles interact with other components of the environment.[44] Some of the basic characteristics of nanoparticles stem to a large extent from their submicron size and, consequently, from their large surface-to-volume ratio.[42] For liposomes and other soluble macromolecular drug carriers, nanoparticles have been shown to be effective in the treatment of certain experimental neoplasic diseases.[6,42,45] This type of vector could be useful in ensuring better peptide delivery.[42]

Typically, the drug is dissolved, entrapped, encapsulated, or attached to a nanoparticle matrix, and depending on the method of preparation, nanoparticles, nanospheres, or nanocapsules can be obtained. Nanocapsules are vesicular systems in which the drug is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed.[36] There are several techniques for preparing nanoparticles, but two methods prevail: dispersion of the preformed polymers[36] and polymerization of monomers.[36,46]

Regarding the use of nanoparticles, the inclusion within the nanoparticles of magnetic particles to direct them to their target (e.g., tumor cells) through magnetic fields created around the tumor brings great advantages, such as the reduction of the dosage and side effects, and raises the therapeutic effect.

15.3 BONE AND CARTILAGE CARRIER SYSTEMS FOR TISSUE ENGINEERING

Bone and cartilage repair is the main goal for several bone and cartilage tissue engineering studies. Their particular properties make it extremely difficult to create *in vitro* a system that mimics in perfection these two tissues. The major challenge in developing an optimal delivery system continues to be the conflicting design elements desired in such a system.[47] In cartilage, the limited regenerative

capacity is one of the main challenges that researchers have to overcome. In bone, the combination of bone tissue with different properties, together with the balance between bone formation and resorption, signaling molecules, and recruitment of bone cells — and in cartilage the particular nature of the tissue together with the cell type — create several barriers to the development of a perfect substitute. Among the many tissues in the human body, bone has been considered as a powerful marker for regeneration, and its formation serves as a prototype for tissue engineering based on morphogenesis.[48] There are many site-specific drug delivery strategies, but osseous tissues are still difficult to target because of the biological and mechanical properties of bone.[49] Osseous tissues have a great amount of the inorganic compound hydroxylapatite (HA), and bones lack the efficient circulatory systems of other tissues, with blood flow rates in bone of 0.05 to 0.2 ml min.1 g.1.[49] As far as scaffold designing and cellular studies are concerned, they are not the scope of this chapter, but they can be found in other chapters of this book. Within this chapter, we will deal with strategies to deliver bone and cartilage factors (from drugs to growth and differentiation factors) that may help or enhance the repair or regeneration of the target tissue for tissue engineering applications.

As it is now well accepted, a tissue-engineered implant is a biological/biomaterials combination in which some component of tissue has been combined with biomaterials to create a device for the restoration or modification of tissue or organ function.[50] The tissue engineering approach is one ideal strategy to help combat serious problems (such as transplantation due to the short number of donor tissues and organs) and to enable the self-healing potential of the patient to regenerate body tissue and organs (in this case, bone remodeling). The development of this specific approach of tissue engineering is based on several observations:[51–53] (1) most of the tissues undergo constant remodeling due to apoptosis and renewal of constituent cells; (2) isolated cells tend toward forming tissue structures *in vitro* if the conditions are favorable; and (3) although isolated cells have the capacity to remodel and form the proper tissue structures, they require a template to guide their organization into the proper architecture.[51] So, according to these observations and in order to achieve a successful bone tissue engineering approach, there are three necessary key components:[51–54] scaffolds, cells, and growth factors, the last one being the main focus of this chapter, although we will also discuss the technology of cell encapsulation as it can be included as a strategy of drug delivery.

For applications that require the creation of large volumes of bone, an optimal carrier would be both a controlled-release system and a scaffold.[47] Additional crucial requirements for the carrier include the ease of manufacture (feasible scale-up of bench processes), cost-effectiveness, biocompatibility, malleability (to fit in various defect sizes), and user-friendliness.[47] In the ideal situation, good carrier selection leads to a synergistic effect with the growth factor.

For the first generation of carriers, researchers frequently turned to the primary constituents of bone matrix — hydroxylapatite (HA) and collagen — because they naturally bind and sequester endogenous BMPs.[48] In addition to collagen and HA, researchers have explored biomaterials with demonstrated osteoconductive properties.[47] In this chapter, biodegradable polymeric systems will be the main focus, with an emphasis on the more recent developments.

Natural materials have been the focus of interest for several groups, and particularly starch-based[2,55–57] and chitosan-based materials[58–60] have been developed in our research group. These materials have been shown to possess properties that can render them suitable for several applications, ranging from scaffolds for bone and cartilage tissue engineering[61] to bone cements[62] and drug delivery systems.[2,63,64] This last point is of particular interest for this chapter, and so far the work from our group has ranged from the encapsulation of anti-inflammatory agents[2] to antibiotics,[63] retinoic acid,[58] and steroids (prednisolone and dexamethasone).[65,66] The materials used are starch-based and chitosan-based materials, both from natural origin.

With regards to cartilage tissue, joint pain as a result of cartilage degeneration due to osteoarthritis is an extremely prevalent age-related disease that causes considerable morbidity,[67] and meniscus lesions are among the most frequent injuries in orthopedic practice.[68] Both cases involve damage at a cartilage level. Since Hunter's observation in 1743 that cartilage once "destroyed, is not repaired," there are a number of therapies possible for articular cartilage repair (for a detailed

review, please see Hunziker[69]), but none have had complete success. The most promising one that will be focused on in this chapter is tissue engineering, specifically the strategies of controlled delivery that can be applied in this therapy (for a review of the tissue engineering approach for articular cartilage, please see Temenoff and Mikos,[70] Barron and Pandit,[71] and van der Kraan et al.[72]). The same combinatorial approaches applied for bone tissue engineering are valid for cartilage tissue engineering, meaning that three necessary components are needed: cells for the generation of tissue, a scaffold to support growth and that degrades as the extracellular matrix is generated, and a bioactive factor to stimulate the correct biological signals *in vivo* for tissue growth and integration with native cartilage. The regulatory effects of growth factors and cytokines in cartilage are well documented.[70] Interesting alternatives seem to be bilayer transplants consisting of periosteal cells for the reconstruction of subchondral bone and chondrocytes for reconstitution of the superficial cartilage layer, or the directed application of growth factors[69] with or within the scaffolds.

The bilayer approach leads us to the discussion of another challenge to be solved involving bone and cartilage. The artificial cartilage prepared from tissue engineering approaches seems to offer promising treatments for cartilage defects.[73–75] The same can be said of the bone tissue engineering approaches that are being developed worldwide.[76–80] However, connecting the soft tissue to bone is difficult, meaning that it is rather complex to develop an osteochondral approach. The natural interface between cartilage and bone contains a zone of calcified cartilage.[73,81,82] Mimicking this calcified interface may be a key issue for adhering an artificial cartilage to bone. One approach is to develop a substrate that supports the growth and attachment of cartilage and encourages a calcified zone. In addition, this substrate should also bond to bone on implantation in order to create an engineered interface between cartilage and bone. Several strategies for treating osteochondral defects are being investigated, such as biphasic transplants,[83] porous polymer–ceramic composites,[81,82,84] anatomically shaped tissue constructs (bilayer),[85] and two-phase composite materials.[86]

As an outline, for bone and cartilage tissue engineering approaches, the scaffold materials and procedures should allow both cartilage and bone regeneration and absorption of the biomaterials over time, so the approach to be used is the application of biodegradable polymers and when necessary, bioabsorbable ceramics to produce the desired scaffolds for this applications. But before the ideal tissue-engineered constructs for bone and cartilage applications are available, many questions remain to be answered — for example, the optimal cell type, the source of the cells, the need for growth factors, and another very important issue, the type of scaffold. From our point of view, the optimal scaffold must be used for stimulation of the cells into the desired tissue, meaning it must be able to differentiate and proliferate the desired cells with the help of the controlled release of relevant growth factor and be able to support the tissue regeneration when implanted and gradually fade out, ideally up to the complete regeneration of the new tissue.[54,87] At this point, we have defined the main factors necessary to achieve this tissue engineering approach: (1) cells, (2) growth factors, and (3) their scaffolds. In other words, it is necessary to increase the number of cells that constitute the tissue as well as reconstruct the structure to support the cells. In addition, growth factors are required to promote cell differentiation and proliferation and then achieve tissue regeneration.[52] The controlled-release concepts can be inherent and be a potential assistant in the three main factors. The "traditional" drug delivery approach can be applied to encapsulate living cells for incorporation within the scaffolds. In turn, scaffolds can be designed as "traditional" drug delivery carriers to control a site- and time-specific release profile and also to protect the growth factor.[87] These strategies will be discussed in the coming sections, together with other potential approaches.

15.3.1 Bone and Cartilage Biologically Active Factors and Strategies for Delivery

Ideally, the combination of an adequate scaffold with proliferating cells and growth factors is the strategy to follow. The steps in between are the most difficult. Several factors are known to act in

TABLE 15.1
Most Widely Studied Molecules Acting on Bone and Cartilage

	Bone and Cartilage Acting Factors	Ref.
TGF-β family	Bone morphogenetic proteins (BMPs)	92–105
	Cartilage-inducing factors A and B (CIF-A and CIF-B)	93, 94
Nonsteroidal anti-inflammatory drugs (NSAIDs)		106–113
Antibiotics		114–118
Biphosphonates		33, 119–124

bone and cartilage, and it should be kept in mind that molecules do not act by themselves to trigger the reaction; instead, triggering of signaling cascades that involve several cellular mechanisms can be involved, and thus, synergistic or antagonistic effects can take place.[88–91] Table 15.1 summarizes the most-studied molecules, ranging from drugs to growth factors, when thinking about bone and cartilage repair and regeneration.

These factors have been so far used in delivery systems to promote the regeneration and repair of bony and cartilaginous tissues. The three major strategies (from a biologically active agent point of view) of delivering bone and cartilage regenerating factors include the following: (1) delivery of DNA encoding a growth factor; (2) *ex vivo* gene therapy (or cell therapy); and (3) delivery of the protein itself via some type of carrier matrix, which has advanced the furthest toward availability as a viable therapeutic.[47] The pretreatment of cells with specific growth factors alone or combined is also a methodology currently used in the research of tissue engineering applications. In the following sections, a brief description of each factor is presented, in addition to the latest technologies for incorporating these factors in the bone and cartilage tissue engineering field.

15.3.1.1 TGF-β Family

TGF-β is a polypeptide that is produced by articular chondrocytes and is present in the cartilage matrix in a latent form.[125,126] Studies have shown that TGF-β increases the synthesis of collagen, proteoglycan, and inhibitors of matrix breakdown and also stimulates cell proliferation.[127,128] Cay et al.[129] tested calcium alginate beads for the delivery of TGF-β in the treatment of osteochondral defects in the rabbit knee and found that this system is capable of sustained release of TGF-β at a steady rate for several days. This system could be applied with other growth factors, such as bone morphogenetic proteins (BMPs). Holland et al.[130] studied the *in vitro* release of transforming growth factor-β1 (TGF-β1) from injectable hydrogels based on oligo(poly[ethylene glycol] fumarate) (OPF). These hydrogels were used to encapsulate TGF-β1-loaded gelatin microparticles and can be crosslinked at physiological conditions within a clinically relevant time period.[130] *In vitro* TGF-β1 release studies demonstrated that reducing the mesh size can reduce burst release from OPF hydrogels, and the encapsulation of TGF-β1-loaded gelatin microparticles within the hydrogel reduced even further the burst. Likewise, final cumulative release after 28 days was reduced from 71% to 48–66% by encapsulation of loaded microparticles.[130] These results indicate that TGF-β1 release can be controlled by adjusting OPF formulation and microparticle loading, which creates a great potential for these materials to be used in the delivery of TGF-β1 articular cartilage defects. This is one promising strategy for encapsulating microparticles within tissue engineering scaffolds, as it will be further discussed later. The microparticles can be used to encapsulate growth factors or even cells. Another example is the work by Peter et al.[131] where TGF-β1 is encapsulated in poly(*D,L*-lactic-co-glycolic acid) (PLGA) and poly(ethylene glycol) (PEG) microparticles and then the influence of this growth factor is studied on marrow stromal osteoblasts cultured on poly(propylene fumarate) (PPF) substrates. It was shown that the TGF-β1 released from PLGA/PEG blend microparticles enhanced the proliferation and osteoblastic differentiation of marrow stromal cells

cultured on PPF substrates, as indicated by the increased total cell number, alkaline phosphatase (ALP) activity, and osteocalcin production over a period of 21 days.[131]

15.3.1.2 Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs), multifunctional cytokines that were originally identified as molecules that induce bone and cartilage formation *in vivo*,[132] are a subfamily of the TGF-β superfamily. In general, BMP-4, -6, and -7 appear to enhance the chondrogenic phenotype.[93] For bone, BMP-2, -4, and -7 seem to have the more prominent effect. In order to increase the efficacy of this potent protein for application in medicine, a carrier system is needed to retain the BMP at the preferred site.[132] Since BMPs act locally, they must be delivered directly to the site of regeneration via a carrier matrix.[47] Many types of carriers have been used to deliver bone growth factors.[133–135] Recently, Weber et al.[132] presented and characterized a slow-release carrier system for pure human recombinant (rh)BMP. The biodegradable carriers consist of large porous microspheres, which the authors call foamspheres, consist of poly(lactide-co-glycolide) acids, and release loaded rhBMP slowly and continuously. *In vivo* studies in rodents revealed that rhBMP-loaded foamspheres increased the thickness of the calvarial bone of rats by 222%. When the same amount of rhBMP was applied via a gelatin-based hydrogel, the increase in bone height was only 66%.[132] Another approach by Saito et al.[136] showed that poly-*D,L*-lactic acid–polyethylene glycol (PLA–PEG) block copolymers exhibited an exquisite temperature-dependent liquid–semisolid transition, enabling them to be used as an injectable delivery system for rhBMP-2.[136] The thermosensitive property of the PLA–PEG/rhBMP-2 composite is permissive to percutaneous injection when heated.[136] The fluidity of this composite decreases as it cools down to body temperature, and the resultant semisolid form provides a scaffold for bone formation through the gradual local release of the rhBMP-2.[136] This new type of injectable osteoinductive material enables a less invasive approach to surgeries involving the restoration or repair of bone tissues.[136]

Microsphere drug encapsulation offers a noninvasive means of delivering growth factors and other regulatory molecules to degenerative cartilage.[137] Unlike scaffolds, microparticles may be injected into defects to provide controlled drug release[137] or even be coupled to the scaffolds to provide sustained release. Microspheres of *N*,*N*-dicarboxymethyl chitosan (DCMC), a copolymer of *N*-acetylglucosamine and glucosamine, have been formulated and impregnated with BMP-7 to regulate the inflammatory response in full-thickness rabbit articular cartilage lesions.[93] Delivery of BMP-7 was able to stimulate chondrocyte proliferation and reduce vascularization and the influx of fibroblast-like cells into these lesions.[137]

Collagen materials have been used as implantable carriers for bone-inducing proteins, such as rhBMP-2.[138] The main applications of collagen as drug delivery systems are collagen shields in ophthalmology, sponges for burns/wounds, mini-pellets and tablets for protein delivery, gel formulation in combination with liposomes for sustained drug delivery, controlling material for transdermal delivery, nanoparticles for gene delivery, and basic matrices for cell culture systems.[48] It was also used for tissue engineering, including skin replacement, bone substitutes, and artificial blood vessels and valves.[48] As examples, the work by Isobe et al.[139,140] and Boyan et al.[141] shows the research that combines the loading of rhBMPs and microspheres with results of osteoinduction *in vivo.*

The critical issue of relative efficacy of various carrier systems for the delivery of BMPs is difficult to address from the general studies, not only because of differences in preclinical models (species, site, dose, etc.) in each study, but also owing to differences in affinity/binding of BMPs to carriers, which can also be an advantage. The localization of BMPs is expected to affect the osteoinductive activity *in vivo,* but this assessment is not possible because the BMP levels at the implant site have not been reported. The studies by Uludag et al.[142,143] were carried out with the ultimate goal of better understanding the relationship between local BMP levels and osteoinductive activity *in vivo.* One interesting aspect of these works is the study of the pharmokinetics of the protein that is normally disclosed is the reported works. For the number and variety of carriers

used, it is recommended to review the work of Uludag.[142,143] The results from these studies indicate that simple application of an rhBMP-2 solution onto an implant could also provide a significant local concentration of the bone-inducing protein,[143] which makes more relevant the application of the desired growth factor encapsulated in microspheres as a potential strategy. The initial recovery of rhBMP-2 was highly variable among the implants, with collagenous sponges retaining the highest fraction of initial dose (70%). There was a gradual loss of rhBMP-2 from the collagenous implants over a 14-day period.[143] rhBMP-2 pharmacokinetics was not affected by differences in the source of collagen (i.e., reconstituted sponges versus bone-derived particles). Mineral and mineral-containing carriers except synthetic hydroxyapatite retained a fraction of implanted rhBMP-2 for a long time in these experiments.[143] These variations in protein pharmacokinetics among different carriers, in addition to variations in their physicochemical nature, are expected to affect the final activity of implanted osteoinductive factors, making more important the study and correlation between all the relevant factors for tissue engineering applications.

15.3.1.3 Osteoprotegerin/Osteoclastogenesis Inhibitory Factor (OPG/OCIF)

Osteoclastogenesis inhibitory factor (OCIF), also called osteoprotegerin (OPG), is a secreted member of the TNF receptor family.[144] In *in vitro* studies, OPG/OCIF inhibited osteoclastogenesis by interrupting the intercellular signaling between osteoblastic stromal cells and osteoclast progenitors.[145] In normal rats, systemic administration of recombinant OPG/OCIF (rOPG/rOCIF) resulted in a marked increase in bone mineral density associated with a decrease in the number of active osteoclasts.[145] Moreover, rOPG/rOCIF prevented bone loss and restored bone strength in ovariectomized rats.[144] Hakeda et al.[146] demonstrated also that OCIF directly inhibits osteoclast function to restrain their functional bone-resorbing activity. On the other hand, OPG/OCIF knockout mice exhibited severe osteoporosis due to enhanced osteoclastogenesis.[144] However, administration of OPG/OCIF prevented the cartilage destruction in adjuvant-induced arthritis in rats,[147] as demonstrated by Kong et al.[147] It was shown that OPG ligand (OPGL)/osteoclast differentiation factor (ODF) expressed in activated T cells is a key regulator of joint destruction and bone loss in adjuvant-induced arthritis in rats and that inhibition of OPGL/ODF activity by administration of OPG/OCIF prevented cartilage destruction and preserved the integrity of cartilage.[147] Adjuvant-induced arthritis mimics pathologic features of human rheumatoid arthritis, which apparently differs from those of osteoarthritis. However, these investigators also detected the expression of OPGL/ODF in T cells isolated from the joints of humans with osteoarthritis, suggesting that OPG/OCIF may also play a role in the pathophysiology of osteoarthritis.[147] Given that osteoarthritis is characterized by progressive deterioration of the cartilage of joints, the increase in the concentration of OPG/OCIF in synovial fluid of individuals with knee osteoarthritis might reflect a compensatory response by chondrocytes or synovial fibroblasts to destabilize the coupling between the degradation and synthesis of articular cartilage. The increased concentration of OPG/OCIF might thus serve to protect cartilage rather than be a cause of osteoarthritis. However, the balance between OPG/OCIF and OPGL/ODF may be an important determinant of bone resorption and cartilage deterioration.[148]

These synergistic effects and the exact role of this growth factor in bone and cartilage may be the reasons why this growth/differentiation factor is not being currently applied in research for controlled-release systems for bone and cartilage tissue engineering applications. However, it seems to be a potential biologically active agent to be applied in the future when the function of these factors in the bone and cartilage tissues is completely elucidated.

15.3.1.4 Cartilage-Inducing Factors A and B (CIF-A and CIF-B)

Although only a few millimeters in thickness, articular cartilage possesses a complex structure, allowing this tissue to absorb and dissipate mechanical shock in joints.[93] The primary components of healthy articular cartilage — water, collagen, and proteoglycans — are contained in a highly ordered extracellular matrix maintained by a sparse population of 10% (weight of articular cartilage)

chondrocytes.[93,149] At skeletal maturity, articular cartilage is the only remaining cartilaginous portion of the long bones.[93] The transforming growth factor (TGF) family includes a number of polypeptides involved in the regulation of cell growth and differentiation and in wound healing.[93] The TGF superfamily also includes two cartilage-specific proteins, cartilage-inducing factors A and B (CIF-A and CIF-B), which promote mesenchymal stem cell (MSC) differentiation along the chondrogenic phenotype.[93] To achieve sustained release, biodegradable osteochondral implants composed of 50:50 poly(lactic acid-co-gylcolic acid) (PLGA) polymers have been examined as drug carriers[93] by implantation in full thickness defects in rabbits. Although the mechanical properties of the systems were worse than the normal tissue, they have been shown to increase the quality of the tissue by 50%.

There are also a number of cartilage-derived growth factors that were already identified to promote the chondrocyte differentiation and proliferation, such as cartilage-derived growth factor (CDGF).[150] However, CDGF was later found to be identical to the ubiquitous fibroblast growth factor-2 (FGF-2).[150] Therefore, cartilage contains intrinsic FGF-2, and this factor has been suggested to be important during chondrocyte growth. Nevertheless, Susuki's group[150] has found some somatomedin-like factors in fetal bovine cartilage, which they named cartilage-derived factor (CDF).[150] In the meantime, TGF-β and other members of its family, such as bone morphogenetic protein (BMP) and cartilage-derived morphogenetic protein (CDMP-1 and CDMP-2), were identified in cartilage and shown to be active in chondrocyte differentiation.[150,151] They then succeeded in identifying the structures of unique cartilage-generated modulators of both chondrogenesis and subsequent osteogenesis, and they named these chondromodulin-I (ChM-I), -II (ChM-II), and -III (ChM-III).[150] For more detailed information on this topic, please consult the paper by Suzuki et al.[150] Because it is not yet widely accepted, it is not easy to find in the literature carrier systems loaded with these growth factors applied to cartilage tissue engineering.

15.3.1.5 Insulin

Investigation of novel experimental application systems for growth factors or other bioactive substances in tissue engineering is often limited by high costs of substances and would benefit from a defined and easily controllable model tissue system.[152] Insulin is an economically viable and potent agent to improve cartilage tissue engineering applications. Insulin can stimulate the growth of a number of cells including chondrocytes.[153] At high concentrations insulin binds to and activates the insulin-like factor-1 (IGF-1) receptor, thus mimicking the effects of IGF-1 itself,[154] which was shown already to have the potential to induce cartilage repair.[72]

Kellner et al.[152] demonstrate a potential three-dimensional *in vitro* system using engineered cartilage as a model tissue and readily available insulin as a model drug. The observed effects of insulin were similar to effects of IGF-I (0.05 μg/ml) and were in agreement with the reported binding constants of IGF-I and insulin at the IGF-I receptor.[152]

Furthermore, Cai et al.[154] studied the effects of a slow-release formulation of insulin in the treatment of osteoarthritis. It was shown that by increasing the amount of matrix retained in cartilage, insulin treatment *in vivo* could lead to maintenance of articular cartilage matrix, and thus inhibition of subsequent joint destruction and deformity.[154] As such, insulin, when delivered in a sustained-release manner, is very attractive as a possible therapeutic for arthritic patients.

Besides the possibility of employing insulin as a potent substance to improve tissue-engineered cartilage, several easily controllable *in vitro* systems were already developed and may be used in the future to evaluate experimental growth factor application scaffolds using economically favorable insulin as a model protein.

15.3.1.6 Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely employed as painkillers.[49] However, NSAIDs have several undesirable side effects, in particular gastrointestinal toxicity.[49] The NSAID diclofenac is used clinically in the treatment of rheumatism,[1,49] but to obtain therapeutic effects,

large doses of diclofenac, which are known to cause gastrointestinal damage, are required.[49] The bone-specific delivery of NSAIDs, therefore, could be beneficial in the treatment of bone disease, while decreasing undesirable gastrointestinal side effects.[49]

Hirabayashi et al.[112] have synthesized osteotropic diclofenac with biphosphonic moiety (DIC-BP) based on the concept of Osteotropic Drug Delivery System (ODDS) and investigated its potency of site-specific and controlled delivery of diclofenac to the bone in rats. DIC-BP once incorporated in the bone was gradually eliminated, releasing diclofenac into the bone compartment. As a result, the bone concentration of regenerated diclofenac was apparently constant over 28 days. DIC-BP exhibited no side effects of gastrointestinal damage, typical of nonsteroidal anti-inflammatory drugs, as it was already stated. Thus, ODDS of diclofenac shows promise as an approach for highly potent and nontoxic therapy of diclofenac with less frequent medication, as it was shown in this study.[112]

Our group also studied the incorporation of an NSAID (meclofenamic sodium salt) in biodegradable starch-based scaffolds.[2] It was shown by the *in vitro* release studies that there was an initial burst release (which can be very useful to treat the first stage of an inflammation process) followed by a controlled release over several days. In addition, similar starch-based materials have already shown the potential to be used as scaffolds for tissue engineering.[61,80,155,156]

15.3.1.7 Antibiotics

Several bone diseases, such as osteomyelitis, are treated with antibiotics. Several antibiotics, such as gentamicin, are known to act on bone diseases. Gentamicin-loaded nanoparticles produced from chitosan and starch–chitosan of natural origin have shown a good release profile for up to 40 days,[58] and these systems can find application in disorders where a prolonged and steady release of antibiotics is necessary. In a study by Yenice et al.,[157] teicoplanin, a glycopeptide antibiotic, was incorporated in a natural biodegradable polymer, alginate, in order to prepare bead formulations for implantation purpose in bone for the localized treatment of osteomyelitis.[157] The system showed a fast release associated with the maintenance of the biological activity of the compound, which is of extreme importance when using release systems, since the loss of the biological activity of the incorporated compound would render the system useless.

15.3.1.8 Biphosphonates

Geminal biphosphonates are a class of drugs that are considered to be stable analogues of pyrophosphate (P–O–P),[158] a physiological regulator of osteocalcification and bone resorption.[158] Biphosphonates have been approved for clinical use in Paget's hypercalcemia of malignancy and in osteoporosis.[159] The affinity of biphosphonates to bone mineral hydroxylapatite (HA)[49,159] is the basis for their use as inhibitors of ectopic calcification and of bone resorption.[159] The physicochemical effects of biphosphonates include binding strongly to the calcium phosphate crystals, inhibiting their growth, aggregation, and dissolution.[160,161] It is now generally accepted that biphosphonates inhibit osteoclast activity[159] by increasing osteoclast apoptosis.[162] Oral route remains the preferred administration delivery method, but with biphosphonates, their reduced absorption rate in the gastrointestinal system renders this route impracticable for their delivery.[159]

Patashnik et al.[163] developed chitosan-based microspheres containing pamidronate and suberoyl biphosphonate for treating several pathologies associated with bone resorption.[163] Chitosan, a widely used natural polymer, seems highly suitable for use in implantable drug delivery systems for treating bone diseases since calcium phosphates can precipitate onto chitosan, serving as a bone matrix while also releasing antiresorption agents facilitating bone repair.[159]

Prodrug strategies have considerable potential as site-specific drug delivery systems.[49] As it was already mentioned, Hirabayashi et al.[112] have recently proposed a drug-delivery system that targets osseous tissues based on a biphosphonic prodrug moiety to release diclofenac. Fujisaki et al.[164] recently proposed an osteotropic drug delivery system based on a biphosphonic prodrug

concept as a novel method for site-specific and controlled delivery of other drugs to the bone,[159] since biphosphonates have a high affinity for bone hydroxylapatite.

15.3.1.9 Other Agents

A recent study showed that addition of 500 IU of retinoic acid to collagen at a site of a bone defect enhanced regeneration of new bone, achieving union across the defect and leading to its complete repair.[165] Our group[63] has developed loaded chitosan nanoparticles with retinoic acid and have shown that these systems possess a long sustained release.

Another important issue when discussing transplant methodologies or approaches is immunosuppression. In fact, immunosuppression is needed in transplantation procedures in order to avoid rejection of the allogenic tissue. The use of allogenic cells requires long-term immunosuppressive therapy.[166] In bone repair, allogenic grafting has not always led to good clinical outcomes, partly because of immunological problems. However, it has the advantage that a larger amount of bone can be harvested.[166]

Nevertheless, a study by Yoshikawa et al.[166] showed that when allogenic bone marrow cells were implanted in Fisher rats, they showed a major incompatibility. FK-506 (an immunosuppressor factor used for hepatic and renal transplantation) needed to be administered in this case after 4 to 8 weeks; the cells displayed a high level of osteoblastic activity, and immunity was well suppressed.[166] Well-known immunosuppressor drugs include ciclosporin,[167,168] rapamicin,[169] and the most widely used FK506,[169–171] a potent macrolide.

15.3.2 Other Polymeric Systems as Potential Approaches for Tissue Engineering

Potential strategies using polymeric systems for bone and cartilage tissue engineering applications currently under investigation have been the focus of this chapter when discussing bone and cartilage growth factors, but there are a number of recent approaches that should also be discussed, even if some are not being used so far as current carrier systems in those applications. They are discussed herein as potential strategies for successful tissue regeneration.

15.3.2.1 Cell Encapsulation

Cell therapy can be considered an *ex vivo* gene therapy as the gene encoding for therapeutic protein is transfected into cell lines or primary cells in culture that can then be encapsulated secreting factor delivered to the desired site.[47] Encapsulated cell technology (ECT) is a rising field of biotechnology that has emerged and developed rapidly in the past decade.[172] Encapsulating cells in a semipermeable material may prevent adverse immune response.[172] There has been significant improvement in the development and optimization of construct technologies directed to produce and characterize new 3-D systems used to release and protect live cells. Current live-cell encapsulation techniques must take into account several key issues[172]: mechanical stability, complete encapsulation and selective permeability for immune isolation, and suitable extracellular microenvironment for optimal cellular functions. Optimization of one issue may sacrifice the other. For example, a thick membrane with good immune isolation and mechanical stability often leads to poor nutrient/oxygen supply for cellular functions.[173] There must be synergetic effects among all the issues, making this technology a challenge in the biomaterials field.

Cell therapy involving transplantation of the progenitors of a given cell lineage is useful for the reconstruction of many types of tissues.[173] With the application of this method for bone tissues, undifferentiated mesenchymal cells such as bone marrow stromal cells are collected beforehand from the patient and are cultivated *in vitro*, differentiated into osteogenetic cells, and proliferated.[174] With this process, the addition of BMP to the culture cells makes efficient differentiation and proliferation possible.[135,173]

Hydrogel scaffolds are appealing for cell delivery and tissue development because they are highly hydrated three-dimensional networks of polymers that provide a place for cells to adhere, proliferate, and differentiate.[175] Hydrogels, three-dimensional networks of hydrophilic polymers that are able to swell large amounts of water, can be made to resemble the physical characteristics of soft tissues.[176] They can also provide chemical signals to the cells through the incorporation of growth factors and mechanical signals by manipulation of the mechanical properties of the material.[175] Hydrogels have a similar macromolecular structure to cartilage, which is a highly hydrated tissue composed of chondrocytes embedded in type II collagen and glycosaminoglycans (GAGs).[175] Thus, cartilage is an obvious tissue to engineer using hydrogel scaffolds. Furthermore, photopolymerizing hydrogel systems provide a method to encapsulate cells and implant materials in a minimally invasive manner.[176]

Photopolymerizations are widely used in medicine to create polymer networks for use in applications such as bone restorations and coatings for artificial implants.[177] These photopolymerizations occur by directly exposing materials to light in "open" environments such as the oral cavity or during invasive procedures such as surgery. Light, which penetrates tissue including skin, could cause a photopolymerization indirectly.[177] Transdermal photopolymerization potentially could be used to create a variety of new, minimally invasive surgical procedures in applications ranging from plastic and orthopedic surgery to tissue engineering and drug delivery.

There are several works for the encapsulation of chondrocytes where photopolymerizing hydrogel systems are applied to achieve a successful encapsulation.[178–180] The work by Elisseef et al.[178] describes the *in vitro* analysis of bovine and ovine chondrocytes encapsulated in a poly(ethylene oxide) dimethacrylate and poly(ethylene glycol) (PEG) semi-interpenetrating network using a photopolymerization process. One day after encapsulation, MTT assay and light microscopy showed chondrocyte survival and a dispersed cell population composed of ovoid and elongated cells.[178] Biochemical analysis demonstrated proteoglycan and collagen contents that increased over 2 weeks of static incubation. Cell content of the gels initially decreased and stabilized. Biomechanical analysis demonstrated the presence of a functional extracellular matrix with equilibrium moduli, dynamic stiffness, and streaming potentials that increased with time.[178] These findings suggest the feasibility of photopolymerization for cell encapsulation in tissue engineering and drug delivery purposes.

Understanding the role of gel properties in extracellular matrix (ECM) formation is important for numerous tissue engineering applications. In the contribution by Bryant et al.,[180] chondrocytes were encapsulated in both degrading and nondegrading gels, and the biochemical composition and distribution of the ECM were examined as a function of the gel properties. When using hydrogel scaffolds for cartilage tissue engineering, two gel properties are particularly important: the equilibrium water content and the compressive modulus. It was found that chondrocytes photoencapsulated in PEG gels with a range of equilibrium swelling and compressive moduli produce a biochemical content similar to that of newly synthesized cartilaginous tissue. However, to obtain a uniform distribution of proteoglycans throughout the gel, the mesh size must be greater than ~ 90 Å.[180] As the gel degrades and the mesh size increases, the proteoglycans are then able to diffuse throughout the gel. By proper design of partially degrading hydrogels, one can maximize the initial gel mechanics without compromising the final distribution of proteoglycans. In the same way but in another study,[179] it was demonstrated that slight variations in hydrogel chemistry control gel degradation, evolving macroscopic properties, and ultimately the secretion and distribution of extracellular matrix molecules. Specifically, biodegradable poly(ethylene glycol)-co-poly(lactic acid) hydrogels were fabricated via photopolymerization for chondrocyte encapsulation.[179] By tuning scaffold chemistry, and subsequently, gel structure and degradation behavior, it is possible to tailor and guide tissue evolution and development.

Photoencapsulation of growth factors containing microspheres in PEO-based hydrogels also proved to be an effective method to deliver these molecules in porous hydrogel systems to enhance the ability to engineer tissues.[181] IGF-I and TGF-β were loaded into PLGA microspheres using a

double emulsion technique.[181] The growth factors containing microspheres were then photoencapsulated with bovine articular chondrocytes in PEO-based hydrogels and incubated *in vitro* for 2 weeks. Microspheres caused an increase in glycosaminoglycan (GAG) production after the *in vitro* incubation,[181] proving themselves to be a potential strategy in cartilage tissue engineering.

For osteoblast encapsulation, Burdick and Anseth[182] have developed a hydrogel system for photoencapsulation of osteoblasts based in RGD-modified PEG to assess their applicability in promoting bone tissue engineering. Nonadhesive hydrogels were modified with adhesive Arg-Gly-Asp (RGD) peptide sequences to facilitate the adhesion, spreading, and, consequently, cytoskeletal organization of rat calvarial osteoblasts. Highly swollen hydrogels are capable of suspending cells three-dimensionally and supporting nutrient diffusion to encapsulated cells, but may not provide an ideal environment for anchorage-dependent osteoblasts. Specifically, the attachment of various peptides to biomaterial surfaces has enhanced the adhesion of osteoblasts and may promote an enhanced biomimetic environment for encapsulated cells suspended in three-dimensional hydrogels. The most commonly researched adhesive peptide, Arg-Gly-Asp (RGD), is found in cell-binding domains of extracellular matrix proteins.[182] Integrins on the surface of cells bind to the RGD and allow cells to adhere to otherwise nonadhesive surfaces. Osteoblasts were successfully photoencapsulated in PEG hydrogels and remained viable, depending on the initial macromer concentration. As a final investigation of this procedure for bone tissue engineering, encapsulated osteoblasts formed a mineralized matrix, which was enhanced in gels containing the adhesive peptides. These systems have potential as an injectable *in situ* forming material that could be reacted via a transdermal photopolymerization.[182]

Mikos' group has investigated[183–185] bone tissue engineering through the use of injectable, biodegradable, polymeric bone cement containing an osteogenic cell population. It is envisioned that the osteogenic cells will initiate a regeneration cascade that will lead to bone formation and eventual replacement of the degradable polymer. A successful cell delivery method for this application must meet several design criteria[183]:

1. The cell carrier must preserve the viability and phenotypic expression of the cell population during composite crosslinking.
2. The cell carrier must maintain its mechanical integrity during injection and crosslinking of the composite.
3. The cell carrier must allow the cells to attach and function in their new environment following the completion of the composite crosslinking.
4. The cell delivery system should only contain the cells for a very short time (1 h or less) before releasing them to interact with their surroundings.

Successful cell delivery meeting all the design criteria can be achieved through the temporary encapsulation of cells in gelatin microparticles. The cells incorporated into an injectable polymeric bone substitute only need to be protected from the local environment during the polymer crosslinking reaction.[183] The experiments demonstrate that marrow stromal osteoblasts did survive temporary encapsulation in gelatin microparticles and that the cells retained their proliferative potential and phenotypic expression.[183] The experiments demonstrate that the cells also survived encapsulation in crosslinked gelatin microparticles.[183] The effect of temporary encapsulation of rat marrow stromal osteoblasts in crosslinked gelatin microparticles on cell viability and proliferation was investigated in this study for microparticles placed on a crosslinking poly(propylene fumarate) (PPF) composite over a 7-day period.[184] Temporary encapsulation of marrow stromal osteoblasts was effective in increasing viability and proliferation of the cells placed on an actively crosslinking PPF composite. The addition time of cells (both encapsulated and nonencapsulated) to a crosslinking PPF composite had a large effect on cell viability and proliferation. Cells that were added to crosslinking composites later had higher viability.[184] There were no indications that the presence of gelatin microparticles affected the crosslinking of the PPF composite.[184] Furthermore, the study investigated the effect of

temporary encapsulation of rat marrow stromal osteoblasts in crosslinked gelatin microparticles on long-term cell proliferation and phenotypic expression for microparticles placed on crosslinking PPF composites using *N*-vinyl pyrollidinone (*N*-VP) as a crosslinking agent over a 28-day period.[185] The results showed that encapsulated marrow stromal cells exhibited much higher viability, proliferation, and phenotypic expression when placed on crosslinking PPF composites than nonencapsulated cells.[185]

Recently, much attention has also been given to peptide-amphiphiles for their ability to produce novel supramolecular structures by a self-assembly mechanism.[186,187] Molecular self-assembly consists of the spontaneous organization of molecules under thermodynamic equilibrium conditions into structurally well-defined and rather stable arrangements, mediated by weak noncovalent interactions such as hydrogen bonds, electrostatic, hydrophobic, and van der Waals interactions.[187] Basically, these peptides are composed of alternating ionic hydrophilic and hydrophobic amino acid residues, which can form complementary ionic bonds and hydrophobic interactions between the regular repeating units.[186] Certain polypeptide sequences have shown the ability to self-assemble or gel in a short period of time by the addition of salt,[186,188,189] and this property may be suitable for the encapsulation of cells or biomolecules (growth factors, enzymes, etc.) within a 3-D gel matrix.

In conclusion, cell therapy by transplantation of progenitors of a certain cell lineage is a powerful approach for reconstruction of tissue, including the skeleton. Although clinical application of autologous transplantation of bone marrow cells (which includes osteogenic cells) to bone repair has been conducted, more suitable procedures of cell therapy are required.[190] Transplantation of osteo/chondroprogenitor cells with biomaterials and local factors stimulating osteoblast or chondrocyte differentiation will provide a new approach to cell therapy of osteo and chondral defects.

15.3.2.1.1 Microparticles with Cells Producing Growth Factors

The encapsulation of cells synthesizing growth factors within carriers targeted to specific tissues is still in its preliminary steps. The viability of cells during the processing step is of great concern, since cells must be maintained at a homeostatic environment during the whole process.[173] Organic solvents are deleterious to cells, and factors such as temperature, pH, ionic composition, and nutrient diffusion are parameters to be considered when dealing with cell encapsulation.[173]

Laurencin et al.[191] have developed a composite delivery system based in poly(lactide-co-glycolide) (PLAGA) and hydroxyapatite for bone regeneration. The polymeric-ceramics system is used as a cellular vehicle for delivery of BMP-2, constructed through retroviral gene transfer.[191] It was found that the composite scaffold supported the BMP-2 production, allowing the attachment and growth of the BMP-2-producing cells.[191] *In vivo*, the scaffold induced bone formation, showing that the system is a delivery vehicle for BMP-2.[191]

15.3.2.2 Coatings

As already discussed, a critical issue in tissue engineering is local and well-timed delivery of the various cell-signaling molecules that are crucial for tissue development. Various approaches have already been presented aiming to combine growth factors and scaffolds. Whereas the development of controlled delivery systems from scaffolds has focused on entrapping the molecule inside the scaffold polymer matrix itself (alone or via encapsulation into microspheres), one interesting approach is to coat the inner pores of a prefabricated scaffold with a bioactive agent-loaded polymeric film, instead of entrapping the bioactive agent inside the scaffold matrix during fabrication of the scaffold.

Sohier et al.[192] have developed a novel emulsion-coating method to obtain the controlled release of proteins from macroporous polymeric scaffolds. In this process, a water-in-oil emulsion, from an aqueous protein solution and a polymer solution, is forced through a prefabricated scaffold by applying a vacuum. After solvent evaporation, a polymer film, containing the protein, is deposited on the porous scaffold surface. Macroporous scaffolds were prefabricated by compression mold-

ing/salt leaching. A microporous, homogeneous protein-loaded coating was obtained on the scaffold surface. Due to the coating, the scaffold porosity was decreased, whereas the pore interconnection was increased.[192] A model protein (lysozyme) was effectively released in a controlled fashion from the scaffolds. Complete lysozyme release can be achieved within 3 days up to more than 2 months by adjusting the coated emulsion parameters.[192] In addition, the coating process did not reduce the enzymatic activity. This new proposed method appears to be an interesting approach for tissue engineering applications, if the changes in the porosity are controlled.

Practically, the hydroxylapatite (HA) porous materials have been used as bone scaffolds to provide improved bone ingrowth and osseointegration.[193–195] However, the brittleness and low strength limited their wider applications in hard-tissue implants. To be used effectively in load-bearing applications, the mechanical properties of the HA porous scaffolds should be improved. Moreover, as a drug delivery system, the pore structure of the scaffold needs to be controlled in terms of porosity and pore size. More importantly, drugs should be entrapped efficiently to be released for a prolonged period.

In order to optimize these requirements, coating design of the porous ceramic scaffold was proposed by Kim et al.[196] By coating with a polymer layer, poly(ε-caprolactone) (PCL), the drugs may be efficiently entrapped through the scaffold by being coupled with polymer. In order to improve the biocompatibility of the polymer, HA powder was hybridized with the polymer. The HA–PCL composites were effectively coated on HA porous scaffolds to optimize the biocompatibility of the system.[196] As drug delivery usage, the antibiotic tetracycline hydrochloride (TCH) was entrapped within the coating layer. Coatings were obtained by a dipping–drying process while varying the concentration and HA/PCL ratio. The loading amount of coatings increased with increasing the concentration and HA/PCL ratio.[196] The composite coatings improved the mechanical properties of the scaffolds, such as compressive strength and elastic modulus.[196] The dissolution rate of the scaffold was well controlled with the coating conditions, and the rate increased with increasing the concentration and HA/PCL ratio. After a free drug release within a short period (≈ 2 h), the drug was released in a sustained manner, and the release rate was highly dependent on the coating dissolution.[196]

Our research group has been studying the incorporation of proteins and enzymes into calcium phosphate coatings on the surface of starch-based polymer using a biomimetic technique[197] (please see Chapter 2.2 by Leonor et al. in this book). This methodology proved to be useful in preserving the activities of enzymes, indicating the carrier potential of calcium phosphate coatings for the sustained delivery of other bioactive agents.[197] These results support the new perspective of adding osteoinductive proteins or growth factors into biomimetic coatings for inducing bone growth on the implant surroundings.[197]

This coating methodology seems an interesting strategy for cases where, for instance, the methods used to prepare polymeric scaffolds are not suitable for the incorporation of thermosensitive biologically active agents, such as proteins, due to the high temperatures used in the processing. Furthermore, it can be used in cases where the scaffold processing involves exposure to organic solvents or interaction with gas or methods such as porogen leaching, which causes the premature loss of protein during the leaching process.

15.3.2.3 Stimuli-Responsive Polymers

Response to a stimulus is a basic process of living systems. Based on this fact, researchers have been designing materials that respond to external stimuli such as temperature, pH, light, electric and magnetic field, chemicals, and ionic strength.[198] Stimuli-responsive polymers are polymers that respond with dramatic property changes to small changes in the environment.[199] This property change can be one of shape, hydrophilicity/hydrophobicity, surface characteristic, solubility, formation of an intricate molecular self-assembly, or a sol-to-gel formation.[198]

In the particular case of pH responsiveness, hydrogels have been widely applied.[200–202] In networks containing weakly acidic or basic pendent groups, water sorption can result in ionization of these pendent groups, depending on the solution pH and ionic composition. For ionic gels containing weakly acidic pendent groups, the equilibrium degree of swelling increases as the pH of external solution increases. Inversely, for gels containing weakly basic pendent groups, the equilibrium swelling degree increases as the pH decreases, making it possible in this way to control the release profile of the carrier.

Our group has been developing pH-responsive chitosan and chitosan/HA scaffolds processed by a microsphere-based aggregation route.[60] At low pH, the chitosan is soluble (pKa of amine group is in the 5.5 to 6.5 range), with the sol-gel transition occurring at approximate pH 7. This pH sensitivity, coupled with the reactivity of amine groups, obtained morphology, and the promising mechanical properties, makes chitosan scaffolds processed by this particle aggregation processing route very promising systems for controlled delivery of bioactive agents in tissue engineering applications.

In another study involving chitosan, Risbud et al.[202] described the synthesis of pH-sensitive chitosan–polyvinyl pyrrolidone (PVP) hydrogels as a controlled-release system of a model biologically active compound. These systems have the particularity of being porous systems. The pH dependency is explained by the pH-dependent swelling behavior due to protonation of the NH_2 group of chitosan that ensures chain relaxation, leading to faster hydrogen bonding dissociation and efficient solvent diffusion in acidic mediums. This will obviously lead to a faster drug release. Again using chitosan, Park and his coworkers[200] developed a novel organic–inorganic composite interpenetrating network based in chitosan and tetra ethyl ortho silicate (TEOS) that has a pH-responsive behavior for the delivery of several model drugs. The optimal TEOS–chitosan ratio for maximum pH-sensitivity was studied, and drug permeation was influenced not only with the external pH, but also with the ionic interactions between the drug and carrier. They also studied the swelling response to rapid changes, showing that these systems present a rapid and also reverse response immediately when local pH conditions are altered. This reversible behavior is very interesting and was also shown for acrylic hydrogels.[201] Furthermore, in this case, the developed hydrogels also present a temperature-sensitive behavior as they are produced with *N*-isopropylacrylamide (NiPAM).

In fact, much interest has been focused on thermosensitive hydrogels made of crosslinked NiPAM that undergo a sharp volume transition at a determined temperature. Amphiphilic polymers with the right balance between hydrophilic and hydrophobic moieties can undergo reversible phase separation or precipitation.[203] The temperature at which the phase separation occurs is called the lower critical solution temperature (LCST). Below LCST, the water is bound to the hydrophilic moieties through hydrogen bonding, and the presence of hydration water prevents the interaction between hydrophobic moieties so that the polymer exists in an extended coil. Above the LCST, the hydrogen bonds are disrupted and water is expelled from the polymer coils, which start to collapse due to the interactions between hydrophobic moieties.[204] The most well known example of a thermosensitive polymer is poly(*N*-isopropylacrylamide) (PNIPA), which presents a sharp phase transition at 32ºC.[204–206] Another polymer with known thermosensitive properties can be obtained by free-radical polymerization of the monomer *N,N'*-dimethylaminoethyl methacrylate (DMAEMA) and presents an LCST of 50ºC in water.[207] In our group, both monomers, DMAEMA and NIPA, were polymerized via a free-radical reaction, in the presence of chitosan.[208] As a consequence of the polymerization process, a semi-interpenetrating polymer network with eventual graft copolymer chains of poly(NIPA) and poly(DMAEMA) onto the chitosan polysaccharide is formed by transfer reactions of the growing radicals on the side substituents of the pyranosyl cycles.[209]

Concerning the release of bone-regenerating factors by thermosensitive polymers, Gao and Uludag[210] have studied the *in vivo* retention of rhBMP-2 entrapped in an *N*-isopropylacrylamide (NiPAM)-based polymer and the influence of their molecular weight on the release of the growth factor. Regarding the polymer molecular weight influence, it was found that molecular weight affects rhBMP-2 release since high molecular weight was essential for rhBMP-2 retention when injected intramuscularly in rats, but not in polymers designed for chemical conjugation with the

growth factor, given that the molecular weight did not make a significant difference in rhBMP-2 retention.[210] The chemical conjugation with the growth factor was achieved with *N*-acryloxysuccinimide-containing polymers.[210] Once again, it is confirmed that using different approaches from a materials science point of view, it is possible to engineer the properties of carriers to meet different treatment methodologies.

Applications involving thermoreversible gels include tissue culture with human chondrocytes, three-dimensional scaffolds, and cell delivery agents.[211] Because the polymer gels at temperatures at or above body temperature, the polymer will solidify when placed in simulated body conditions. The cells can be seeded in the polymer at temperatures below the LCST, or below body temperature. The polymer is in a liquid state, and cells can be thoroughly mixed with the polymer. The cell-seeded polymer can then be placed at temperatures above the LCST to facilitate gelling and solidification. Cells can be grown in a three-dimensional culture mimicking the natural environment of the cells. Propagation of cells in such biocompatible matrices inhibits their tendency to shift to a fibroblastic phenotype *in vitro.* Using the thermoreversible gel allows cells to remain immobilized *in situ* so that the matrix can serve as a vehicle for cell delivery into the tissue defect. Little research has been done to analyze the ability of such thermoreversible polymers to serve as cell scaffolds. These principles were used by Au et al.,[211] who have evaluated a thermoreversible biomaterial to serve as a scaffold for the propagation and amplification of chondrocytes that promotes the original cellular phenotype of these cells.[211] The goal of the study was to investigate the use of thermally reversible polymer gels, poly(NiPAAm-co-AAc), as a biocompatible supporting scaffold for the propagation of chondrocytic cells. The temperature-dependent properties of solutions and gels were investigated using dynamic rheometry methods. It was observed that aqueous solutions of all synthesized copolymers flowed freely at room temperature and formed soft gels at physiological temperature range as a result of a sol–gel transition. The gels were fully reversible, that is, exhibited also a gel–sol transition, and were classified as physical gels.[211] The polymer gels at temperatures above its LCST, whereas it liquefies at temperatures below its LCST of 34.5°C. Hence, the polymer, in its gelled form, has the ability to hold cells *in situ*, forming a matrix similar to the natural cellular environment or the extracellular matrix that composes cartilage. The hypothesis that the polymer gel promotes cell viability and function was tested. Cells propagated in the poly(NIPA-co-AAc) gel-containing culture were found to have an enhanced growth rate compared with cells grown in a monolayer culture.[211] Although it was presumed that the gel matrix would hinder the cells from propagating through the scaffold, cells did proliferate at a greater rate in the three-dimensional culture. The gel allowed for more surface area where the cells could multiply in more than just one layer. Chondrocytes propagated in the thermoreversible polymers expressed enhanced or maintained expression of collagen type II and aggrecan.[211] Collagen type I expression was decreased or unaltered.[211] The *N*-isopropylacrylamide and acrylic acid copolymer gel has potential use as a cell culture substrate and as a cell delivery vehicle. The cell culture method used in this study facilitated the evaluation of candidate biomaterials for cell culture. The polymer gel supports cell viability, growth, and metabolic activity, allowing the copolymer to potentially be used as a reversibly gelling cell culture substrate. The results provide evidence to support the hypothesis that the thermally reversible poly(NIPA-co-AAc) copolymer may be suitable as a cell culture substrate and may have potential use for tissue repair.[211]

In order to synchronize the drug-release profile with physiological conditions, mechanisms responding to physiological variations must be designed. An ideal drug delivery system for tissue engineering applications should respond to physiological requirements, sense the changes, and accordingly alter the bioactive agent release profile.

15.3.2.4 Combining Microspheres and Scaffolds

The incorporation of growth factors into open-pore matrices imposes a great challenge because of the inherent inability of these matrices to efficiently entrap and control the release of macromole-

cules. One way to overcome this challenge is to incorporate microspheres within the scaffold, ideally without interfering with the porous structure of the scaffold.

For bone tissue engineering applications, Kempen et al.[212] present a new approach to fabricate porous poly(propylene fumarate) (PPF) scaffolds with incorporated poly(lactic-co-glycolic acid) microspheres for the release of bioactive molecules. Using a double emulsion–solvent extraction technique, the model drug was encapsulated into poly(lactic-co-glycolic acid) microspheres. Porous scaffolds were fabricated by injecting PPF in a low viscosity state with microspheres into a coated mold using a disposable syringe with needle. A foaming technique was used to create porosity. Compared with microspheres alone, the scaffold–microsphere construct had a lower burst release during the initial phase of release. Scaffold and microspheres exhibited a sustained release of the model drug for at least 28 days.[212]

For cartilage repair, we have already mentioned the work by Holland et al.[130] in which gelatin microspheres were encapsulated within oligo(poly[ethylene glycol] fumarate) (OPF) hydrogels, where the *in vitro* release studies demonstrated that the swelling properties of both the OPF and gelatin components of these biomaterials influence the release kinetics of TGF-β1. Therefore, unique *in vitro* TGF-β1 release profiles may be obtained by altering microparticle loading and OPF formulation and crosslinking time.

Regarding the TGF-β1 growth factor and cartilage applications, Kim et al.[213] designed a novel type of porous chitosan scaffold containing this growth factor encapsulated in microspheres to enhance chondrogenesis. To achieve a sustained release of TGF-β1, chitosan microspheres loaded with TGF-β1 (MS-TGFs) were previously prepared by the emulsion method, in the presence of tripolyphosphate. MS-TGFs were seeded onto the porous chitosan scaffold, prepared by the freeze-drying method, to observe the effect on the proliferation and differentiation of chondrocytes. It was obviously demonstrated from *in vitro* tests that, compared with the scaffold without MS-TGFs, the scaffold containing MS-TGFs significantly augments the cell proliferation and production of extracellular matrix, indicating the role of TGF-β1 released from the microspheres.[213] It was revealed that the sustained release of TGF-β1 from MS-TGF allowed the chondrocytes to rapidly proliferate on the chitosan scaffold.

Another challenge in engineering a thick tissue construct is to provide adequate nutrients and oxygen to the transplanted cells within a few hours after implantation.[214] The environment at the implant site is usually nutrient-poor by virtue of being several cell layers away from the nearby capillaries and is likely to remain so until angiogenesis occurs.[214] Thus, the simultaneous delivery of potent angiogenic agents, such as bFGF, from the tissue-engineered biograft may allow the vascularization to be enhanced. Site-specific delivery of angiogenic growth factors from tissue-engineered devices should provide an efficient means of stimulating localized vessel recruitment to the cell transplants and would ensure cell survival and function. One way of achieving this goal is by incorporating angiogenic factors into the scaffold, such as the potent basic fibroblast growth factor (bFGF), which induces the proliferation of endothelial cells, fibroblasts, smooth muscle cells, and hepatocytes.[214] Perets et al.[214] aimed at exploring and developing 3-D alginate scaffolding for tissue engineering with the capacity for the controlled delivery of bFGF. Basic FGF was first encapsulated within 3-μm-diameter, biodegradable poly(lactic-co-glycolic acid) (PLGA) microspheres, which were then incorporated onto the scaffold during its fabrication.[214] The incorporated microspheres did not interfere with the porous structure of the alginate scaffold, and they were an integral part of the continuous solid wall of the matrix. Such scaffold morphology should enable the penetration of blood vessels throughout the matrix volume, without the bottleneck of interfering microspheres. Furthermore, the retention of 90% porosity in the scaffold should enable a large space for accommodating high-density cell cultures. The controlled delivery of bFGF from the three-dimensional scaffolds accelerated the matrix vascularization, since it induced the formation of large and mature blood vessels.[214] The number of penetrating capillaries into the bFGF-releasing scaffolds was nearly fourfold higher than into the control scaffolds (incorporating microspheric BSA and heparin but not

bFGF).[214] So, in tissue engineering, the incorporation of bFGF into the cell delivery vehicle by means of encapsulating previously into microspheres would ensure tissue survival and function.

One novel methodology to design polymer scaffolds is based in the microsphere technology. The aggregation of polymer microparticles method consists in the agglomeration, by physical or chemical means, of microparticles.[215] This technology is further discussed in Chapter 4.5 in this book by Gomes et al. with a review of some works[216–220] that are being developed with this technology in the scaffold design. The porosity obtained in this type of scaffold can be easily controlled by the microsphere diameter that will create the interstices when the particles are aggregate. If an increased pore size is desired, it is also possible to use macroparticles as it has been done in our group with chitosan-based particles.[60] Chitosan is the product of the partial deacetylation of the naturally occurring polysaccharide chitin, which is found in the exoskeletons of insects and marine invertebrates. It was shown already that this polymer supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes,[221] making chitosan a potential material to be used in tissue engineering for the repair of osseous, chondral, and osteochondral defects, and it has been used for several biomedical applications in our group.[58,59,222] A main advantage of the described microsphere-based technology is the possibility of loading a growth factor within the scaffolds as the microspheres are produced. In this way, the polymeric system acts as carrier and scaffold simultaneously.

15.4 CONCLUSIONS AND FUTURE TRENDS

Drug delivery remains an important challenge in medicine and, more recently, has become a key issue in the tissue engineering field. So far, drug delivery systems have evolved from simple systems to more sophisticated systems, and drug delivery is among the most researched areas in the field of biomedical applications.[223] The drug delivery field is developing at a fast rate, and advances in microfabricated drug delivery systems ranging from transdermal microneedles to implantable microchips[224] are at the present making drug delivery a fast and worldwide developing technology. With regards to bone and cartilage drug delivery, it is easy to recognize its potential for the enhancement of bone and cartilage repair/regeneration, and the variety of systems that can be applied, together with the wide range of bioactive agents to be delivered, creates a whole range of combinations that can be of extreme importance in patient recovery. Of all the studied bioactive agents, BMPs are at the top of the list, and biodegradable systems are clearly the materials to be used. Some studies[87,102,225–229] have shown the benefit of *in vitro* and *in vivo* associations of therapeutic agents such as antibiotics, growth factors, and steroid hormones with bone substitutes. In the future, the association of different therapeutic agents will be an interesting approach for enhancement of the action of these agents over the tissue to be repaired. Microencapsulation of drugs can overcome several problems[230] that may arise, and the combination of micro- or nano-sized systems (such as micro- and nanoparticles) adequate for controlled release with scaffolds that support the structure and cells that can even produce the therapeutic agents themselves is expected to provide the key tools to investigate the ideal approaches for bone and cartilage tissue engineering applications. This will ideally help move forward this research field, moving tissue engineering from the laboratory benches into the hospitals and clinics.

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16 Resorbable Polymeric Delivery Systems Based on Physical Absorption/Diffusion versus Chemically Controlled Delivery Systems

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CONTENTS

16.1 INTRODUCTION

The controlled release of bioactive compounds is a rather simple and logical concept that has attracted the attention of many researchers because of the great importance of delivery systems in relation to the application of drugs in the field of pharmaceutical sciences or the development of the emerging field of tissue engineering. *Controlled release* is a term that represents an increasing number of techniques by which active chemicals are made available to a specified target at a rate and duration designed to accomplish a specific therapeutic effect.[1] There are enough reasons to justify the design, development, and application of controlled-release systems, which are associated to the following main objectives:

- To extend the time that the effective therapeutic doses are present at the target from a single administration, avoiding the toxic effects of overdose.
- To increase the biodisposability of very soluble or insoluble drugs in a particular environment, protecting tissues from secondary effects such as irritation or inflammation.
- To target active drug to the precise point at which it is necessary, avoiding the dissemination, invasion, or spreading of the drug through the whole organism.

- To decrease the toxicity of drugs when they are applied by the traditional way.
- To reach certain zones of the organism not easily available by oral administration or injection of specific drugs. The most representative example is the poor permeability of the sinovial membrane of joints in inflammation processes produced by traumatic accident or illness.

After more than 40 years of research and development of a huge quantity of systems, it is clear that the polymeric supports are decisive in the design and preparation of controlled-delivery formulations. In fact, the great versatility of polymers from a structural point of view, together with the enormous possibilities to combine hydrophobic and hydrophilic components, as well as the interactions between polymer–polymer macromolecules, polymer–drug, polymer–solvent, or polymer–physiological medium, offers infinite possibilities to design and prepare formulations with specific properties and functions. These interactions are regulated by the functional groups and the nature of chain segments of the polymeric systems. According to the macromolecular chemistry concepts, the inter- or intramolecular interactions can be regulated or controlled by one of the following four functions represented schematically in Figure 16.1.

Strong ionic interactions between functional groups with opposite charge are well established. This property is applied for the preparation of a great number of "intelligent systems" sensitive to the pH of the medium, which in addition are capable of retaining drugs bearing ionic groups through the formation of ionic reversible complexes. This principle is the basis for the development of several bioadhesive controlled-delivery systems. For high-molecular-weight polymers, the secondary links through hydrogen bonding or Van der Waals interactions can be even more important than covalent bonds and are the basis of the formation of strong molecular complexes between specific functional groups of macromolecules and drugs. Also, the presence of dipolar groups contributes noticeably to the formation of stable systems that can be programmed for the controlled release of bioactive compounds including drugs, growth factors, and other elements, by means of bioadhesion or by the formation of semipermeable membranes sensitive to the physicochemical characteristics of the surrounding medium (for example pH, temperature, ionic strength, etc.).

Finally, the hydrophobic interactions between nonpolar segments of high-molecular-weight chains can afford specific microdomains that affect the retention of drugs and provide enough stability to control the release of the active system. All these factors contribute to the cellular and tissue organization in the human body, starting with the well-known specific and nonspecific interactions of proteins, which are responsible for the biochemical activity of cells.

According to these principles, there are two large categories of design and preparation of controlled-delivery systems considering the structure and functions of the polymeric supports. These are classified as "physically controlled delivery systems" based on the physical interactions of the bioactive components with the support, and "polymeric drugs" based on the chemical link of the bioactive component to the polymeric support. According to the site of action, both of them can be considered as "controlled drug release systems" and "targeted drug delivery systems," depending on the relationship between the site of drug release and the site of drug action. Controlled-release systems deliver drug into the systemic circulation at a predetermined rate, whereas targeted delivery systems release the bioactive component at or near the site of action, providing a high concentration of drug just at the site where it is needed without dissemination over other parts of the body.[2,3]

The main objective in the design and preparation of a controlled drug delivery system is to release a bioactive compound in a predetermined, predictable, and reproducible fashion. This is considered on the basis that the selected bioactive agent exhibits fewer side effects when its concentration in circulation is kept constant at some optimum level over prolonged periods of time.[4] However, it has been clearly demonstrated that in many applications constant drug concentrations are not necessarily the best treatment, and in this context the modulated release of bioactive compounds has been considered for the design of delivery systems sensitive to external stimuli,

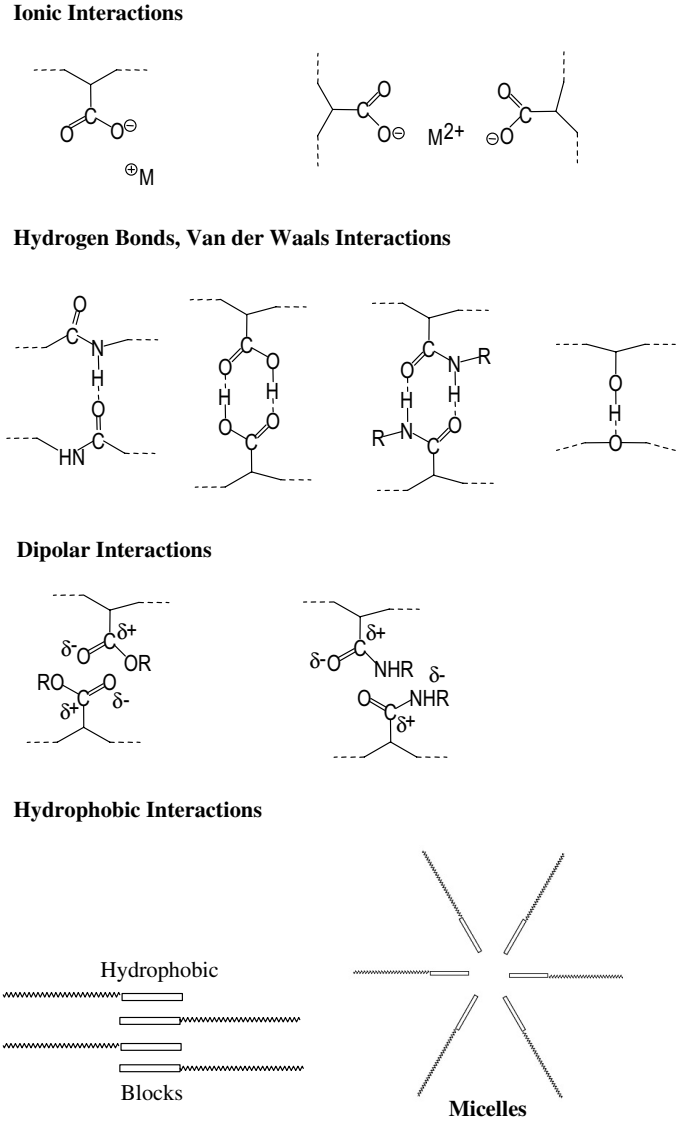


FIGURE 16.1 Intra- and intermolecular interactions in polymer systems.

which have been called "smart supports." These are polymeric chains sensitive to metabolites, pH changes, temperature, magnetic or electric fields, ultrasound or microwave irradiation, etc.[5]

One of the most interesting characteristics of modern controlled-delivery systems is the use of specific polymers, which offers a considerable degree of freedom in the choice of their site of action. Traditional formulations might be injected or ingested, but new polymeric systems can be placed into available body cavities or can be applied as mucoadhesives or as transdermal delivery compositions by the temporary attachment of the complex system to the wall mucosa or the skin. In addition, they can be implanted subdermally or just in the cavity in which the action profiting by the surgical operation is necessary.

In this chapter, we present the most important characteristics and differences between controlled-delivery systems based on physicochemical interactions of polymeric matrices with bioactive compounds and those based on the chemical control of the release mechanism as a

consequence of the labile covalent link of the bioactive component to the polymer matrix. Examples of both kinds of systems with applications in drug release, based on solution/diffusion process, as well as the action of specific "polymer drugs," are presented as a result of a cooperative work of specialists in different areas of material science, pharmacy, medicine, and biology. According to the mechanism of release of the bioactive product (drug, growth factors, hormone, etc.), the controlled-release formulations can be considered as "solvent controlled," "diffusion controlled," and "chemically controlled" systems. This classification considers that the rate of release is controlled predominantly by solvent interactions such as swelling of the polymer chains, by the diffusion of drug through a polymer matrix or a membrane, or by chemical processes such as polymer degradation, erosion, or the cleavage of the bioactive compound chemically linked to the polymeric support. It is clear that these mechanisms operate simultaneously in most practical situations and cannot be considered separately.

16.2 SOLVENT-CONTROLLED SYSTEMS

The main physical principles involved in solvent-controlled systems are swelling and osmosis. Most of the swelling-controlled systems are hydrogels and the rate of drug release depends on the hydrophilic/hydrophobic character of the polymeric matrix in the case of uncrosslinked systems, or the cross-linking density in the case of network structures. Peppas,[6] Okano,[7] and Hoffman[8] have published excellent reviews on the preparation and properties of hydrogels for biomedical applications. In these systems, the hydration-induced swelling of the polymeric system produces the activation of the release process. If the bioactive component is homogeneously dispersed in the polymer matrix, the release will be produced when the interactions of the penetrating solvent with the polymer and the drug compensate the interactions of the polymer matrix with the drug as well as the polymer–polymer interactions. In general, the original dry systems are stiff and become flexible when the hydration process is produced. In addition, the hydration process generally makes the polymer–drug delivery system mucoadhesive, with a relatively good adhesion to the tissues surfaces in contact with the system. One problem of this kind of system is the clearance of the polymeric support from the body after the release of the bioactive compound. This clearance is difficult to achieve when biostable crosslinked polymeric chains compose the matrix. Therefore, to avoid the effect of the support accumulation, two different ways have been suggested. One is based on the use of biodegradable polymeric chains derived from natural polymers, mainly polysaccharides such as dextrane, starch, chitin, or chitosan, or proteins such as collagen and polypeptides such as poly(*L*-lysine). The second alternative is the use of noncrosslinked polymeric systems with a controlled character of the hydrophilia. In this case, the release of the bioactive compound is produced after the hydration process, and simultaneously the resorption of the polymeric support is produced by the slow dissolution of the polymeric matrix in the medium. In this case, it is not necessary that the polymeric support be degraded in the medium, if the molecular weight of the system is low enough to cross the membrane of the kidney to be incorporated into the excretion products.

We have studied recently some applications of noncrosslinked hydrogels of controlled hydrophilic/hydrophobic character based on copolymers of vinyl pyrrolidone (VP) with 2-hydroxyethyl methacrylate (HEMA) prepared by free-radical polymerization. VP-HEMA random copolymers of molecular weight between 35 and 40 kDa were obtained.[9] In these supports, the release is controlled by the solubilization rate of the matrix after a relatively fast swelling process, as initially described by Narasimhan and Peppas,[10] who performed a very deep analysis of the process using PVA as crystalline polymer model.

Poly(VP-co-HEMA) is a well-known biocompatible hydrogel with broad applications in the biomedical area,[11,12] mainly as crosslinked networks. The most characteristic use is probably as contact lens support material. On the other hand, because of its biocompatibility and hydrophilic nature, it has been investigated as a carrier for drug delivery.[13] However, crosslinked matrices are

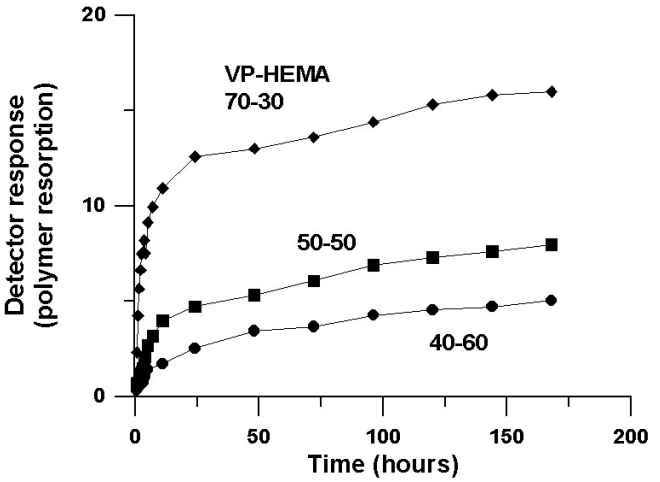
not resorbable. In this sense, we have proposed the use of noncrosslinked VP-HEMA copolymers as entirely resorbable delivery systems. As a consequence of its relatively high hydrophilia, this copolymer has been shown to be successful for the release of drugs such as cyclosporine with very low solubility in water[14] and for the release of macromolecules such as growth hormone (GH).[15] Cyclosporine (cyclosporine A) is a cyclic oligopeptide that has been shown to be very successful as an immunosuppressor. However, its use is associated with serious toxic side effects, primarily renal and hepatic.[16] On this basis, a control in the release of this compound is very interesting. On the other hand, recombinant growth hormone controlled-delivery systems might be very helpful in therapeutic treatments for tissue regeneration associated with some pathologies. Typical examples are the regeneration of bone tissue or the healing of persistent ulcers in diabetic patients. Due to the low stability of GH in physiological media (its half-life is a few hours), a polymeric depot loaded with the hormone can be used not only as a controlled-delivery system of the protein, but also as a protective matrix. The correlation between the solubilization and the release rate is shown in Figure 16.2. Cyclosporine release rate and polymer dissolution are depicted versus time for some *in vitro* experiments. Further *in vivo* experiments using rat as animal model exhibited a very good correlation with this previous *in vitro* data.[17] The most hydrophobic implant reverts a provoked immune response slower (2–4 weeks) than the most hydrophilic one (1–2 weeks), which agrees with the release timescales obtained from the *in vitro* experiments.

The solubilization properties of this VP-HEMA copolymer depend on the hydrophilic and hydrophobic balance associated to the VP/HEMA composition, respectively. This balance can be tailored in the synthetic procedure profiting from the particular characteristic of this polymerization process. Basically, HEMA is much more reactive than VP, and therefore the copolymer microstructure formed in the course of the reaction can be considered as a blend of (1) a HEMA-rich copolymer with an average composition regulated by the initial molar ratio in the feed, and (2) a VP-rich copolymer formed in the last steps of the polymerization when most of the more reactive HEMA has been consumed. The control on the feed and the conversion leads to a control on the species ratio and on the properties of the final materials. In this way, we have been able to obtain solubilization rates (*in vitro*) ranging from a few hours to several months.

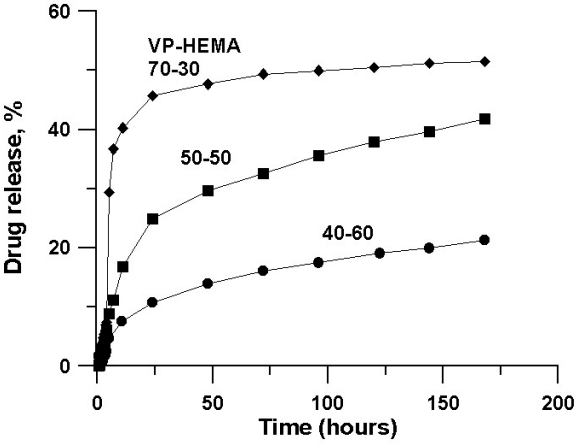
According to the microstructure of copolymer chains, the poly(VP-co-HEMA) systems prepared at high conversion could be considered as interpenetrating physical networks constituted by chains very rich in VP, which are very soluble in water, entangled into chains of random copolymers of VP-HEMA, which are less soluble but highly hydrated in water or physiological fluids. This is schematically drawn in Figure 16.3 in which the bold lines correspond to the VP-rich polymer chains. After the hydration process, these chains become very soluble in the hydrated medium, becoming incorporated into the solution and giving rise to the formation of microchannels that favor the release of the drug or the bioactive component loaded into the polymer matrix. These structures formed during the free-radical polymerization of the VP and HEMA comonomers modulate the release of the bioactive component and the resorption of the hydrogel within periods of time up to 4 or 6 weeks, showing a very good biocompatibility.

The preparation of this kind of controlled-delivery system is very easy because of the solubility of the copolymeric matrix in common organic solvents compatible with the bioactive compound. It is possible to prepare powder by lyophilization, or films by casting from water/dioxane solutions. In any case, when the system is in contact with the hydrated medium, it swells very fast to become a mucoadhesive gel, which releases the drug and becomes resorbable simultaneously.

Amphiphilic block copolymers composed of hydrophilic and hydrophobic segments are promising candidates as drug supports. Poly(methoxy poly[ethylene glycol]-co-ε-caprolactone), for example, can form micellar structures, with a hydrophobic compact inner core and a hydrophilic swollen outer shell in selective solvents, and it has been investigated for targeting drugs to specific sites.[18] Since most drugs have a hydrophobic character, they can be easily incorporated into the micelle core by a covalent or a noncovalent bonding by dialysis or solvent evaporation methods, among others.



(a)



(b)

FIGURE 16.2 (a) Polymer dissolution and (b) cyclosporine release rate, depicted versus time for some *in vitro* experiments of VP-HEMA copolymers 70-30, 50-50, and 40-60 wt%, respectively. (From Gallardo, A. et al., *Biomaterials*, 21, 915, 2000. With permission.)

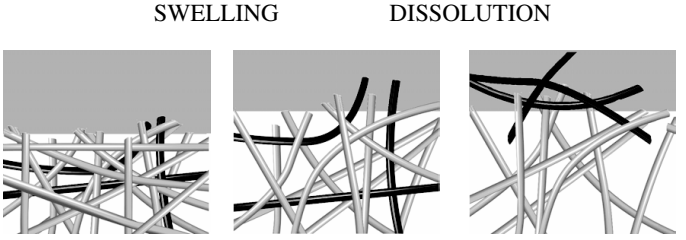


FIGURE 16.3 Schematic swelling and differential dissolution of the poly(VP-co-HEMA) matrices.

In addition, temperature- or pH-sensitive polymers have been extensively applied to drug delivery systems. Several systems, such as poly(ethylene oxide) (PEO)–aliphatic polyester diblock copolymers and PEO–aliphatic polyester–PEO, show a sol-gel transition by increasing the temperature of aqueous solutions. Several biodegradable aliphatic polyesters, such as poly(*L*-lactic acid) (PLLA), poly(*D,L*-lactic acid) (PDLA), poly(lactic acid-co-glycolic acid) (PLGA), poly(DLLA-co-PCL), etc., have also been used. Recently, new systems containing block copolymers with amphiphilic character (i.e., pluronics [PEO-PPO-PEO] as the hydrophilic component and PCL as the hydrophobic component) have been evaluated as potential drug carriers for drug delivery systems.[19] These materials exhibit a temperature-responsive behavior and sustained release of certain model drugs. Multiblock copolymers composed of PEO and PCL were reported to display a thermoreversible sol-gel transition in aqueous solutions close to body temperature.[20] The transition results from the melting of PCL domains, whose chains are associated by hydrophobic interactions, and the collapse of physical networks. The phase transition behavior was mainly determined by the PEO/PCL block ratio, PCL block length, and the molecular weight. These characteristics may open a way to the design of new controlled-release systems based on phase separation-induced processes.

16.3 DIFFUSION-CONTROLLED SYSTEMS

There are many possibilities for the design of delivery systems in which the release of the active component is produced by diffusion. The selection of a particular system or device depends on the application, but corresponds basically to two general principles of design: reservoir and matrix. The reservoir systems typically consist of a polymeric semipermeable membrane that controls the release of the drug incorporated to an encapsulated core. These systems can be designed as microcapsules, hollow fibers, or tubes of nondegradable polymers, which is a limitation in these pharmacological applications. In these reservoir systems, the drug release is strictly controlled by the diffusion through the polymeric membrane, the preparation being relatively easy and not expensive. One important limitation of these systems is the risk of high concentration of drugs in the core of the device, which makes it necessary for the polymeric membrane to be very stable chemically and mechanically to guarantee the integrity in order to avoid an overdose. This is probably the main reason for the choice of systems based on a matrix design rather than reservoir.

The design of delivery systems based on polymeric matrices with homogeneously distributed drugs, which can be released by diffusion or erosion, is probably one of the most important topics of a great number of researchers in the field of controlled-release technologies. These systems can be fabricated in a variety of geometries, sizes, and shapes, including microspheres that can be injected or self-curing cement formulations. Depending on the type of application, nondegradable or biodegradable polymers can be used. In this section, we analyze several examples of nonbiodegradable cement formulations and biodegradable or partially biodegradable systems designed for different applications. When biodegradable systems are used, the release of the active compound is produced by a double mechanism of diffusion and degradation. In addition, the degradation of the polymer matrix can be produced in bulk or on the surface by erosion. These mechanisms are the limiting factors for the rate of dosage of the drug, with the size and shape of the biodegradable device being important factors for controlling the concentration of the active compound. However, the biodegradable character of the system guarantees the clearance of the matrix from the body.

It is important to take into consideration that when biodegradable systems are used in biomedical formulations, it is necessary that the polymers and their degradation products be nontoxic and the degradation rate be coherent with the mechanism of release of the drug diffusion. On this basis, poly(α-hydroxyacids) have been selected as the most appropriate polymers for the design of biodegradable/diffusion controlled-delivery systems. Nondegradable systems are designed for long-term applications, mainly as a complement to surgical processes when filling material is necessary. Typical applications in dentistry and orthopedic surgery are well documented in the literature and

have become very common, showing very good results. It is necessary to consider that in this kind of system the rate of diffusion decreases with time, showing a first step of a relatively fast release corresponding to the diffusion of the drug present in the most external layers, and a very slow second step corresponding to the inner zone of the device. Therefore, the concentration of the drug is strongly dependent on the surface area of the device.

Current self-curing acrylic formulations for drug delivery systems are mainly based on poly(methyl methacrylate) (PMMA). Gentamicin[21] and other antibiotics[22] are added for both local and systemic treatment. The additives diffuse out of the PMMA in measurable quantities. PMMA beads are also used as a carrier for local implantation of antibiotics. The use of antibiotic-impregnated cement and antibiotic-impregnated PMMA beads has been found effective in the treatment of chronic osteomyelitis and acute musculoskeletal infections, as well as in soft-tissue infections of the neck, abdomen, and rectum.[23] The use of PMMA beads has the disadvantage that once the drug has been delivered, the beads have to be removed in order to avoid foreign body reactions in the wound. In this sense, some experimental biodegradable formulations have been developed. Gerhart et al. prepared a biodegradable bone cement in which a hydrolyzable prepolymer, poly(propylene fumarate) (PPF), was crosslinked with MMA in the presence of antibiotics. After polymerization, the strong material formed degrades subsequently over weeks to months, as the hydrolysis of the prepolymer occurs.[24] These cements loaded with vancomycin have been effective in the prevention of infections in the tibia of rats.[25] Likewise, biodegradable and injectable composites prepared with PPF crosslinked with VP in the presence of sodium chloride and β-tricalcium phosphate have been developed for use as a biodegradable cement in clinical orthopedic applications, especially filling skeletal defects and trabecular bone replacement.[26] Other injectable PPF-based bone cements with osteoconductive properties have been prepared, and the *in vivo* experimentation in rats has demonstrated the osteoconductivity of these types of materials.[27]

Composites based on PMMA/PCL, which can be polymerized *in vivo* and also supply some structural support before degradation, have been prepared by our group as an alternative to the drug delivery systems. The composites were formulated with beads of PMMA/PCL[28] charged with 5 wt% vancomycin.[29] In all cases, release of vancomycin was initially very rapid (burst effect)[30] and slowed down markedly afterward. For the PMMA cement, 20% of the incorporated vancomycin was released in the first 3 h, and thereafter a slow release was observed, leaching 30% of the total initial drug after 50 days. The cement prepared with PMMA/PCL beads of 86/14 eluted 64% of the initial drug within the first 5 h, and afterward it released progressively nearly the total amount of the initial drug (90%) in approximately 2 months.

Other drug delivery systems have been prepared from acrylic resins polymerized in the presence of a bioactive component. New bioactive bone cement consisting of *bis*-GMA/triethylene-glycol dimethacrylate (TEGDMA) (1:1) resin and apatite and wollastonite-containing glass-ceramic powder has been developed and used as the basis of the gentamicin delivery system.[31] Likewise, the controlled release of gentamicin has been studied from poly(ethyl methacrylate)/poly(methyl methacrylate)/hydroxyapatite[32] and from materials prepared from PLLA/PMMA using bioactive glasses as the SiO_2-CaO-P_2O_5 system.[33] We have prepared composites based on PMMA and phosphate glasses (PG) in the system P_2O_5-CaO-Na_2O to be used as carriers of vancomycin controlled delivery.[34] Release profiles were found to be influenced by the content of phosphate glasses present in the cement. The cement containing 47% PG produced an initial release of 30% of the total amount in the first half hour (burst effect), in contrast to the PMMA control in which the amount released in this period of time was close to 17% of the total loaded amount. The cements prepared with 40% and 20% PG released a lower amount of drug, 8% and 6%, respectively, during this period of time. The equilibrium drug release content and time to reach equilibrium increased with increasing phosphate glass content in the composite. For the control, after the burst effect, the drug was eluted very slowly, with only 30% of initial amount being released in 20 days. However, the composites with 47 wt% and 40 wt% bioactive glasses continued releasing the drug at a uniform

rate until about 94% and 60% release, respectively, over a period of 50 days. These differences were attributed to the dissolution of the phosphate glasses[35] in the simulated body fluid.

On the other hand, it is possible to combine the properties of diffusion and partial degradation by the preparation of polymeric systems that contain biostable and biodegradable components in their structure. In this context, we have prepared graft copolymers by radical polymerization of a poly-*L*-lactic acid macromonomer and other acrylic monomers.[36,37] This procedure is useful for obtaining systems with different properties and time of resorption. According to this procedure, when a stable and durable polymeric matrix has to be designed, copolymers of lactic macromonomer and MMA or methyl acrylate (MA) can be used. The degradative behavior can be controlled by the composition, taking into account that, according to the hydrolytic mechanism, the final polymeric residue will be a copolymer of MMA or MA and the salt of methacrylic acid, which becomes soluble in aqueous media under the appropriate conditions, avoiding in this way the accumulation of the macromolecular carrier. Films and microspheres from copolymers of the lactic macromonomer and other hydrophobic acrylic monomers such as MMA or MA and hydrophilic vinyl monomers such as VP were prepared using casting and emulsion/evaporation methods, respectively. Both are monolithic systems but with different shape, size, and surface area. The synthesized systems have been applied as supports of ibuprofen (taken as a model drug). In the case of MMA-LLA and MA-LLA graft copolymers, the release of ibuprofen in buffered solution is modulated by the flexibility of the copolymer chains in a first step of 1 to 2 days, and in a second step by the diffusive properties of the system as well as by the biodegradation of the polymers. The VP-LLA graft copolymers are highly hydrophilic, and the release of ibuprofen is modulated by the diffusion of the drug through the swollen system. Specific interactions between the ibuprofen molecules and the pyrrolidone rings also participate in the kinetic behavior of the release process. Figure 16.4 and Figure 16.5 show the *in vitro* drug release profiles as a function of incubation time, determined by UV spectroscopy, for the films and microspheres described.

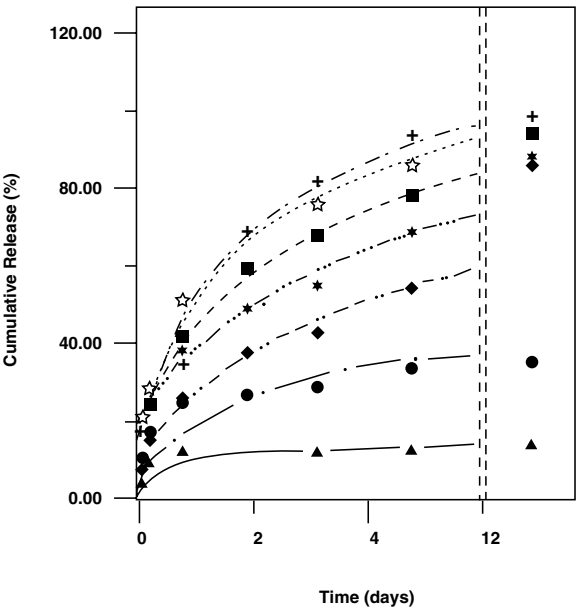


FIGURE 16.4 Ibuprofen release profiles of films of 500 µm thickness. (+) MA29, (♦) MA47, (■) MA66, (●) MMA53, (▲) MMA74, (☆) VP20, (★) VP43. (From Gallardo, A. et al., *J. Control. Rel.*, 55, 171, 1998. With permission.)

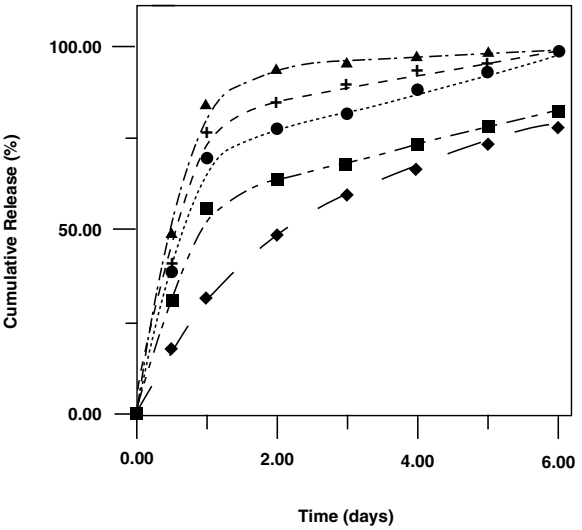


FIGURE 16.5 Ibuprofen release profiles of microspheres. (+) MA29, (♦) MA47, (■) MMA53, (●) MMA74, (▲) VP20. (From Gallardo, A. et al., *J. Control. Rel.*, 55, 171, 1998. With permission.)

One promising support for controlled release of antitumoral agents involves the use of partially biodegradable matrices implanted within the cavity of a rejected brain tumor. Graft copolymers of PCL on poly(dimethylacrylamide) (PDMAm), PMMA, or poly(DMAm-co-MMA) have been synthesized and characterized in our laboratory. These partially biodegradable copolymer matrices are proposed as drug delivery systems for the release of a synthetic carbohydrate able to inhibit the proliferation of human malignant glioma cells in culture and transplanted glioma in rats.[38] The swelling and the degradation rate of the polyester component could be tailored to some extent by the composition of the acrylic part. Figure 16.6 shows the accumulated glycoside release as percentage of total drug initially loaded into the polymer. The drug release seems to be carried out mainly by diffusion through the swollen polymeric matrix better than by biodegradation of PCL-grafted acrylic chains. The combination of hydrophobic/hydrophilic structures can lead to the control of the biodegradation rate of the grafted polyester moieties, and the ratio of the three components can tailor the swelling and degradation rate and then modulate the drug release from the matrix to the tumor bed.

With respect to pure biodegradable systems, as we have previously indicated, polyesters have been widely used as carriers in controlled-release delivery systems due to their known biodegradability and biocompatibility. Numerous efforts have been focused on the optimization of fabrication variables, blending of polymers, and synthesis of copolymers with different compositions to obtain controlled and predictable release profiles. Promising and attractive systems are the poly(β-hydroxyalkanoates) (PHAs) as natural polymers of high molecular weight and narrow distribution that are sensitive to biodegradation and are biocompatible. They are polyesters synthesized by a wide variety of bacterial microorganisms that are accumulated as an intracellular energy and carbon source storage material.[39] Regarding their composition, the structure of PHAs is quite variable as a function of the bacterial strain used to produce them and the culture conditions employed for obtaining these polymers. PHAs usually contain β-hydroxyalkanoic acid monomeric units, with poly(3-hydroxybutyric acid) being the most common and representative polymer. Recently, PHAs have received considerable attention and have shown promise as hard-tissue implant materials because of the combination of unique properties such as piezoelectricity, thermoplasticity, biodegradability, and biocompatibility.[40] Their use as drug delivery vehicles in various forms such as microcapsules and rods has been reported,[41] and their *in vivo* performance, especially on treatment

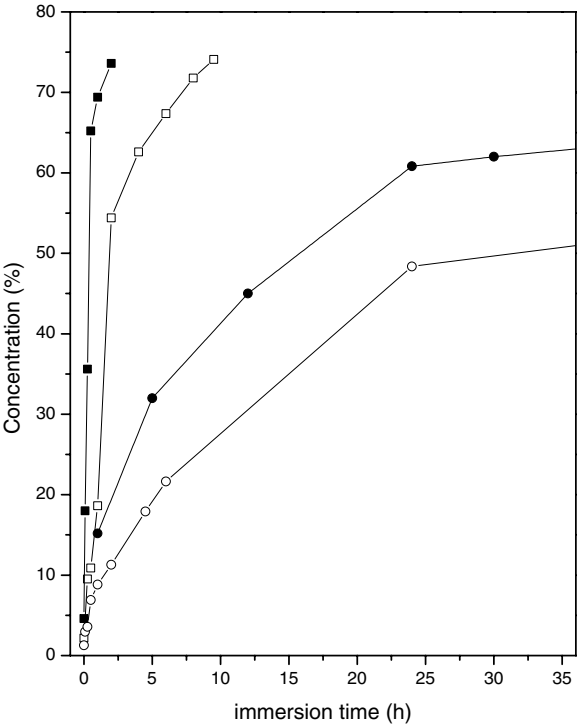


FIGURE 16.6 *In vitro* release profiles of drug-loaded polymer discs for the following samples with composition PCL-DMAm-MMA wt%: (–■–) 60-40-0; (–□–) 56-27-17; (–▲–) 48-8-44; (–O–) 63-0-37. (From Abraham, G.A. et al., *J. Biomed. Mater. Res. Part A,* 64A, 638, 2003. With permission.)

of implant-related osteomyelitis, is also being investigated.[42] Besides these characteristics, some PHAs are remarkable in that they can contain unusual groups in the side chain (halogens, olefins, cyano, nitrile, or methyl ester groups). García et al. have reported that different pseudomonas species (*P. putida* U) are able to synthesize many different PHAs containing unsubstituted phenyl groups (PHPhAs) if cultured properly, and the yields of production can be strongly increased by using some mutants disrupted in the β-oxidation pathway.[43] Recently, we have biosynthesized and characterized different PHPhAs obtained from genetically engineered *P. putida*, when they were cultured in a chemically defined medium containing several ω-phenylderivatives of fatty acids for supporting bacterial growth.[44] These studies open new possibilities for the synthesis of polymers that, depending on their physicochemical properties, could be used for the preparation of new biodegradable and biocompatible carriers for long-term dosage of drugs and bioactive species and in other biomedical applications.[45,46]

16.4 CHEMICALLY CONTROLLED SYSTEMS

Although the biodegradable systems are sensitive to chemical reactions, mainly hydrolysis in hydrated medium, we have considered them in the context of this chapter as combined diffusion/degradation systems in order to differentiate them from the pure chemically controlled systems, which refers to polymers that present the bioactive component covalently linked to the polymeric chain and form a wide and important group known as polymer drugs or polymeric drug delivery systems. These are characterized by the activity of the polymeric system itself as a macromolecular active drug (this activity in the macromolecular form is common in nature, the best example being the pharmacological activity of enzymes, peptide hormones, and proteins). In addition to these

effects, the system behaves as a controlled-delivery support of the corresponding bioactive component and is eliminated from the body by dissolution in the physiological fluids followed by biodegradation or, if the molecular weight of the polymer chains is low enough, by excretion. Excellent reviews of these systems have been published by Hoffman[47] and Kopecek.[48,49]

Synthetic or natural polymers may be conjugated covalently by weak hydrolytically sensitive bonds with a great number of bioactive compounds including drugs, peptides, proteins, growth factors, hormones, enzymes, etc. These possibilities make the polymeric conjugated systems very useful for applications not only in medication but also in tissue engineering, biosensors, affinity separations, enzymatic processes, cell culture, etc. One of the most attractive advantages of synthetic polymers for use in the biomedical field is the great diversity in composition, molecular structure, molecular weight, and molecular weight distribution. In addition, it is possible by copolymerization reactions to control the hydrophilic or hydrophobic character of the conjugate, which makes the system very suitable for specific applications including the design of targeting. There is also the possibility to use reactive conjugation sites at one or both ends of a macromolecule or distributed along the polymer chain as pendant side groups. All these concepts are described in an excellent chapter published recently by Hoffman.

According to these characteristics, an elegant rationalized model of the drug–polymer (chemical) conjugation was proposed by Helmut Ringsdorf in 1975 with three components incorporated within a macromolecular chain: a component chemically linked to the drug (maybe through a spacer), a targeting unit, and a molecule to control the solubility or the hydrophilicity. Since then, numerous conjugated systems have been designed and investigated (as described by Hoffman). Particularly relevant are two conjugates, based on the work of Duncan and Kopecek[50] and Maeda et al.[51] currently used in clinic. The design and application of these conjugates is a field in continuous expansion and development because of the intrinsic advantages offered by specific macromolecular systems in new therapies. The use of polymeric systems with pharmacological activity provides very good local activities reducing the toxicological risks and in addition could act as release systems of the pharmacologically active residue, controlled by chemical reactions, mainly hydrolytic processes under enzymatic catalysis.[48,52] From a chemical point of view, it is possible to prepare macromolecular systems with good initial stability, which guarantees the long-term pharmacological action, and with specific functional groups that, after the hydrolytic process and the release of the pharmacologically active counterpart, allow the clearance of the support due to changes in its solubility.

We have been interested in the preparation and application of families of polymer–drug conjugates based on acrylic derivatives of several compounds with pharmacological and medical interest: vitamin E,[53] antiaggregating drugs such as Triflusal,[54] analgesic compounds such as salicylic acid or paracetamol,[55] and other nonsteroidal anti-inflammatory agents (NSAIDs) based on derivatives of phenyl acetic or propionic acids.[56–58]

16.4.1 Ibuprofen and Ketoprofen Conjugates

Ibuprofen and ketoprofen are two well-known NSAIDs widely used in anti-inflammatory therapy. Their main disadvantages are the relatively short half-life in plasma and a significant gut and nephrotoxicity.[59,60] Therefore, a controlled-release system with hydrophilic character would be useful, especially in chronic diseases such as rheumatoid arthritis. In this sense, several research groups have devoted attention to the preparation of polymeric drugs bearing ibuprofen and comonomeric hydrophilic components.[61–64] Moreover, similar systems have revealed *in vivo* activity in their macromolecular form, that is, high-molecular-weight acrylic polymers incorporating these drugs present a noticeable pharmacological activity.[56] This fact makes these systems quite interesting for local, long-term (intra-articular) applications. In addition, these drug delivery systems are expected to be clearable after hydrolysis because of the formation of the corresponding sodium salts, which are soluble in the physiological fluids.

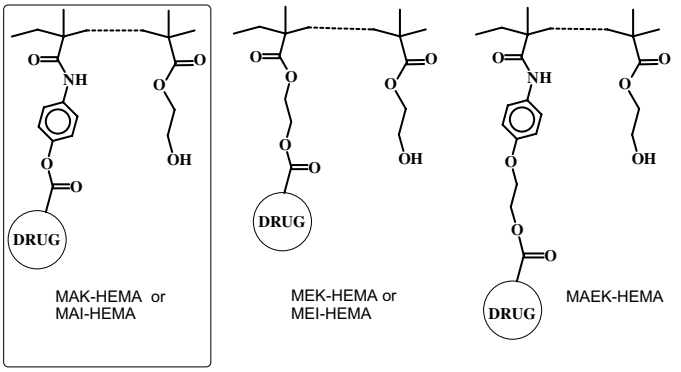


FIGURE 16.7 The structures of the copolymers with 2-hydroxyethyl methacrylate (HEMA), where the drug moiety can be ibuprofen or ketoprofen. Three types of spacers have been considered in the design: aromatic, aliphatic, and aromatic/aliphatic. (From Gallardo, A., Parejo, C., and San Román, J., *J. Control. Rel.*, 71, 127, 2001. With permission.)

The structures of the systems prepared in our laboratory, which are copolymers with HEMA, are presented in Figure 16.7, where the drug moiety can be ibuprofen or ketoprofen. Three types of spacers have been considered in the design: aromatic, aliphatic, and aromatic/aliphatic.

The swelling, hydrolysis, and drug diffusion processes are dependent on the global hydrophilic/hydrophobic balance and flexibility, which are controlled by the chemical structure of the spacer. Small changes in the flexibility or in the hydrophobic character of this spacer will affect the accessibility and the hydrolytic stability of active components, which are important factors in the release behavior of the systems. All the systems can be considered as poly-HEMA derivatives, which is a well-known biocompatible hydrogel with broad applications in the biomedical area.[65–68] The introduction of HEMA molecules allows the control of the hydrophilicity of the global system and the hydrolytic sensitivity by the presence of ester or amide bonds that can overcome hydrolysis in physiological conditions. Therefore, the preparation of these systems provides a good instrument to change the physicochemical properties of the drug (hydrophilic character) as well as its relative stability in physiological conditions, which is related to the targeting effect. We have performed *in vitro* release experiments with copolymers rich in HEMA, from 1 to 30 wt% of active monomer content. In this range, from the microstructural analysis, it has been shown that HEMA units preferentially surround the drug derivatives. These hydrophilic acrylic copolymer systems offer a controlled release in terms of several weeks that can be modulated by the composition of the copolymer system and by the type of spacer.

16.4.2 Biocompatible Conjugates with Antiaggregating Properties for Platelets

The biological response of the human body to the surface of foreign implants and devices in contact with blood is characterized by the activation of the coagulation cascade, the aggregation of platelets, and the formation of thrombus.[69,70] The most generalized method to prevent this phenomenon has been the administration of heparin solution as well as polymeric derivatives of heparin.[71–73] However, in addition to the potent anticoagulant activity, heparin presents side effects such as the promotion of platelet aggregation and blood lipid clearance,[74] which has promoted the direction of research toward the preparation of biocompatible polymeric systems with antithrombogenic properties by the anchorage of residues with intrinsic antithrombogenic activity, such as thrombin inhibitors[75–77] or compounds with antiaggregating effects for platelets.[78,79] In this sense, the antiaggregating properties of aspirin and other derivatives of salicylic acid are well known.[80–82] We have demonstrated that the application of coatings with polymeric derivatives of salicylic acid on the inner

surface of small-diameter Dacron or Gore-Tex vascular grafts improved the prevention of adhesion and aggregation of platelets on the surface of the vascular graft under dynamic conditions.[83,84]

The derivatives of salicylic acid studied were constituted by high-molecular-weight polyacrylic chains (M_n ~ 40,000 Da) bearing the salicylic residue as side substitutes bound to the polymeric chain by weak carboxylic ester functional groups, which are easily hydrolyzed in the physiological medium. In this way, this kind of coating not only presents an intrinsic antiaggregating character, but also acts as a controlled-delivery system of salicylic acid. In addition, after the release of the active residue, the main polymeric chain becomes totally soluble in the physiological fluids, since the sodium salt of polymethacrylic acid constitutes them. The hydrolytical process does not produce the biodegradation of the polyacrylic chains, but changes the solubility of the support, which is cleared readily from the body by the classical metabolic pathway.

Triflusal, 2-acetoxy-4-trifluoromethyl benzoic acid, is a commercial platelet inhibitor with a chemical structure closely related to aspirin and a characteristic pharmacological profile.[85,86] In view of the previous results obtained with polymeric derivatives of salicylic acid, we considered the preparation and application of polyacrylic derivatives of triflusal. This drug, in addition to having the structure of salicylic acid, contains acetyl groups which are considered to be related to the irreversible deactivation of platelets in the aggregation process and, therefore, with the inhibition of cellular thrombus. Two types of polyacrylic derivatives of triflusal — a hydrophobic homopolymer and a hydrophilic copolymer with dimethylacrylamide (DMAm)[54,87] (a well-known biocompatible hydrophilic component that in addition has shown good behavior in contact with blood)[53,88] — have been synthesized from the acrylic derivative of the drug. The preliminary results of aggregation found in static conditions seem to indicate that coating the surface of vascular grafts of Gore-Tex improves the antithrombogenic character of the prostheses and provides a resorbable system that allows the reendothelization of the prosthesis after implantation in a moderate interval of time. In addition, the new polyacrylic systems derived from triflusal are truly controlled-release systems of the antithrombotic drug triflusal, as is shown in Figure 16.8. There is a clear dependence on the hydrophilia of the system, with the release rate of the copolymer being much higher than that exhibited by the hydrophobic homopolymer.

16.4.3 Polymeric Drugs with Antioxidant Properties Derived from Vitamin E

It is well established that lipid peroxidation proceeds through a free-radical mechanism and that the free radicals are involved in damaging processes of cell membranes as well as in cell aging. It seems that the biological antioxidant function of vitamin E *in vivo* is based mainly on the protection of unsaturated lipids of the cells from the damaging effect of peroxidation. We have reported recently the synthesis of a new acrylic derivative of vitamin E that can be polymerized with hydrophilic acrylic or vinyl monomers to obtain hydrogels bearing vitamin E structures as side groups of the high-molecular-weight polymer chains.[89] The acrylic structure of this vitamin E derivative offers a new route for the design and preparation of biomaterials with specific properties. The copolymerization of the acrylic derivative with comonomers such as HEMA, DMAm, or VP provides biocompatible polymeric drugs with controlled hydrophilic character according to the average composition of copolymer chains. The physicochemical properties of these systems are excellent for the application of this copolymer as a powder that after hydration forms a pharmacologically active hydrogel. Particularly we have analyzed the excellent behavior of hydrogels prepared from copolymers of the acrylic derivative of vitamin E and HEMA in the healing process of Achilles tendon of rabbits. The regeneration of the tendon is clearly favored by the presence of the active polymeric system, with a good reorientation of the fibrillar collagen in the longitudinal direction. The histological study of the regenerated tissue demonstrated that the polymeric derivatives of vitamin E stimulate the regenerative process as a consequence of the antiaging effect in the local area of application.

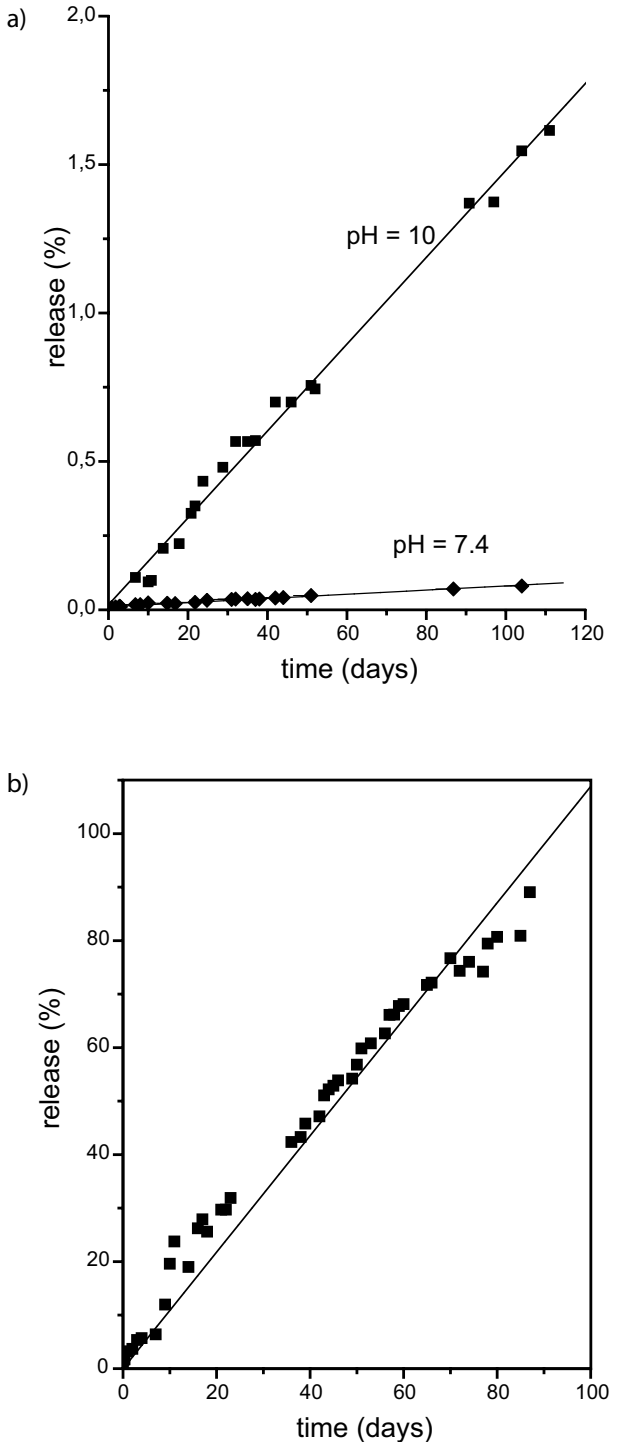


FIGURE 16.8 Cumulative release of the active metabolite HTB (a) from the homopolymeric system as a function of time at pH = 7.4 and 10; (b) from the copolymeric system at pH = 7.4. (Adapted from Rodríguez, G., *J. Mater. Sci.: Mater. Med*., 10, 873, 1999 and Rodríguez, G. et al., *Macromol. Biosci.,* 4, 579, 2004.)

16.5 CONCLUSIONS

It is not easy to establish a comparative criterion between controlled-delivery systems based on physical properties, such as those considered in this chapter, "solvent activated release" or "diffusion/biodegradation activated release," and the "chemically controlled release of polymeric drug delivery systems," since all of them present some advantages and limitations and the applications are generally different.

The physical controlled-delivery systems can be prepared by several techniques, using a number of polymers: linear, branched, or crosslinked systems that can be blended with the active component or processed by techniques such as dispersion in hydrated medium, lyophilization, or the formation of microparticles or microcapsules by dispersion-evaporation methods, spray coating and spray drying, etc. A clean method to be considered in the near future is the use of supercritical fluid technologies, using carbon dioxide as solvent or fluid.

However, it is not easy to avoid the burst effect of the systems prepared, and the release of the bioactive components depends on the hydrophilic character of the matrix and on the biodegradative activity of the medium. It is also necessary to have a good distribution of the drug in the matrix, which is not very easy in the case of crosslinked or stiff hydrophobic polymeric systems.

Many different biomolecules can be chemically incorporated into specific polymer chains with predictable molecular weight and molecular weight distribution. The polymer–drug conjugate can exhibit pharmacological activity as a macromolecule and in addition acts as a controlled-delivery system. Targeting and amplification of the drug action or even new effects can be attained by conjugation of drugs plus targeting molecules to reactive side groups of polymer molecules. The application of polymer–drug conjugates in cancer therapies as well as in gene therapy seems to be some of the most interesting possibilities.

The development of controlled-release systems will offer more interesting strategies for the effective pharmacological application of active compounds, growth factors, proteins, hormones, etc., and will contribute to the development of new emerging technologies such as tissue engineering and gene therapy.

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17 Enzyme Immobilization in Biodegradable Polymers for Biomedical Applications

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CONTENTS

17.1 INTRODUCTION

During the last three decades, enzymology and enzyme technology have progressed considerably and, as a result, there are many examples of industrial applications where enzymes, in the native or immobilized form, are being used. These include food industry, materials processing, textiles, detergents, biochemical and chemical industries, biotechnology, and pharmaceutical uses.[1] The overall impact of enzymes on industrial applications is, however, still quite limited due to their relative instability under operational conditions, which may involve high temperatures, organic solvents, and exposure to other denaturants. Various approaches, including, among others, addition of additives,[2,3] chemical modification,[4,5] protein engineering,[6] and enzyme immobilization,[1,7] have been assessed for their ability to increase the stability of enzymes toward heat or denaturants.[7,8]

The use of enzymes in medical applications has been less extensive as those for other types of industrial applications. For example, pancreatic enzymes have been in use since the nineteenth century for the treatment of digestive disorders. At present, the most successful applications of enzymes in medicine are extracellular, such as topical uses, removal of toxic substances, and the treatment of life-threatening disorders within the blood circulation.[9] The production of therapeutic enzymes has progressed, but the costs of enzyme production, isolation, and purification are still too high to make them available for clinical applications. Furthermore, the ability to store unstable enzymes for long periods of time is also a limitation for their more widespread use. Most applications in the biomedical field are still in the state of basic studies rather than definite applications, owing to the absence of the necessary information on toxicology, hemolysis, allergenicity, immunological reactions, and chemical stability of the system *in vivo*.[9,10]

This chapter will focus on the importance of using enzymes in medical applications and, in particular, the use of immobilized enzymes. For that, some aspects of enzyme immobilization technology, including the traditional physical and chemical methods and new immobilization methodologies, based on biological and genetic engineering approaches, will be reviewed. Several examples will be given of immobilized enzymes in various support materials and using different immobilization strategies, according to the objective of their application in the biomedical field.

17.2 ENZYMES IN MEDICINE

Since the mid-1950s there has been a considerable increase in both measurement of enzyme activities and the use of purified enzymes in clinical practice. In recent years, many enzymes have been isolated and purified, and this made it possible to use enzymes to determine the concentration of substrates and products of clinical importance. A further development, arising from the increased availability of purified enzymes, has been targeted to enzyme therapy.

17.2.1 Clinical Diagnosis

The measurement of enzyme activities in serum is of major importance as an aid in diagnosis, being used as means of monitoring progress after therapy, recovery after surgery, and detection of transplant rejection. Urine can be also analyzed for determination of enzyme activities since the detection of certain enzymes in urine may indicate kidney damage or failure.[11] On the other hand, the concentration of certain metabolites in serum or urine may be determined by enzymatic methods. The method consists of using an enzyme to transform a metabolite into its product and then estimate the amount of transformed substrate. The use of enzymatic methods presents several advantages, such as the high specificity of the enzyme to estimate the concentration of the metabolite in the presence of other substances, avoiding the need of purification steps prior to chemical analysis. In addition, enzymatic reactions are performed at mild conditions, allowing the analysis of labile compounds that would be degraded by harsher chemical methods. The cost of purified enzymes may be, however, too high to support routine analysis, but the use of immobilized enzymes allows for enzyme reuse and the application of immobilized enzymes for diagnostic assays and as biosensors will be further discussed in Section 17.3.2.4 of this chapter. The determination of serum metabolites in serum by enzymatic methods includes a wide range of substances such as glucose, uric acid, urea, cholesterol, cholesterol esters, triglycerides, and creatine, among others.[11]

17.2.2 Enzyme Therapy

Many inborn metabolic disorders are associated with the absence of activity of one particular enzyme normally found in the body. Of the 1250 autosomal recessive human genetic diseases, over 200 involve errors in metabolism that result from specific known enzyme deficiencies.[12] Table 17.1 lists some examples of inborn errors or disorders in metabolism due to enzyme deficiencies.

TABLE 17.1
Some Examples of Inborn Errors and Disorders Associated with Enzyme Deficiencies

Inborn Error/Disorder	Enzyme Deficiency	Frequency (%)	Ref.
Gaucher disease	Glucocerebrosidase	0.003–0.002	11, 13–15
Acatalasemia	Catalase	0.004–0.004	11
Hypophosphatasia	Alkaline phosphatase	≈ 0.001	11, 15
Glycogen storage disease type Ia	Glucose 6-phosphatase	0.001	11, 15
Alkaptonuria	Homogentisate 1,2-dioxygenase	0.0001–0.001	11
Phenylketonuria	Phenylalanine 4-monooxygenase	0.005–0.01	11, 15, 16
Fructosuria	Fructokinase	0.0008	11
Pentosuria	L-Xylulose reductase	0.04 in Ashkenazi Jews	11
Tay-Sachs	β-*N*-acetyl-D-hexosaminidase	0.0003	11, 13–15
Infantile neuronal ceroid lipofuscinosis (INCL)	Palmitoyl protein thioesterase 1 (PPT1)	≈ 0.013 live births	17, 18
Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator	0.03–0.05 Caucasians, rare in other ethnic groups	11
Albinism	Tyrosinase	0.02 schoolchildren in Zimbabwe	16, 19, 20
Glucose-6-phosphate dehydrogenase deficiency	Glucose-6-phosphate dehydrogenase	0.5–26	11, 21, 22
Neonatal jaundice	Biotinidase	0.0025	23, 24
Prolidase deficiency (PD)	Prolidase	—	25
Pompe's disease	α-glucosidase	—	13, 14
Severe combined immunodeficiency (SCID)	Adenosine deaminase	—	12, 15, 26
Xanthinuria	Xanthinine oxidase	—	12

The initial identification of the disease may be difficult to determine and normally requires a tissue biopsy. For some genetic diseases, DNA probes are now available that can be used on small amounts of blood, cells, or amniotic fluid. Albinism, for example, is often caused by the absence of tyrosinase, an enzyme essential for the production of cellular pigments. Tyrosinase is a copper-containing enzyme that catalyzes the first two rate-limiting steps in the melanin biosynthetic pathway, the oxidation of tyrosine to dopa and the subsequent dehydrogenation of dopa to dopaquinone. The human tyrosinase gene, encoding 529 amino acids, consists of five exons spanning more than 50 Kb of DNA in chromosomes.[20] When homozygous mutations of the tyrosine gene result in complete absence of melanogenic activity, such a patient, categorized as tyrosinase-negative oculocutaneous albinism, will never develop any melanin pigment in the skin, hair, and eyes throughout his or her life.[20] Some inborn errors in metabolism are relatively harmless, e.g., albinism or alkaptonuria, but others must be detected early if the defect is to be circumvented. This is the case of phenylketonuria, where the enzyme phenylalanine 4-monooxygenase (enzyme that converts phenylalanine into tyrosine) is missing. Phenylketonuria results in the accumulation of phenylalanine, which may cause mental retardation. Patients with phenylalanine 4-monooxygenase deficiency must follow a phenylalanine-free diet in order to avoid the accumulation of deleterious effects. Phenylalanine is, however, an amino acid essential to maintain growth and protein turnover, and thus it must be supplied in a minimal amount required to maintain normal metabolism. Such a dietary scheme is normally carried out[11,16] by lowering the amount of protein consumed, but this may cause a deficiency in other essential amino acids. A possible alternative to this therapy is to replace the missing enzyme. It may be difficult to find, however, an enzyme with the same function from a human source since the direct administration of enzymes from other sources into the body would cause an adverse immunological response. A possible approach to circumvent this problem

TABLE 17.2
Enzymes with Therapeutic Importance for Medical Applications

Enzyme	Typical Applications	Ref.
Lysozyme	Recommended in treatment of certain ulcers, measles, multiple sclerosis, some skin diseases, and postoperative infections (antibacterial agent)	27–29
Urease	Biosensor and artificial kidneys	30–32
Catalase	Treatment of acatalasemia and removal of hydrogen peroxide in human cells	33–35
Glucose-6-phosphate dehydrogenase	Treatment of jaundice	36–38
Collagenase	Skin ulcers	11
Glucose oxidase	Glucose test in blood and urine	39, 40
Asparaginase	Anticancer agent (leukemia)	41–43
α-amylase, protease, lipase	Digestive aids	44
Chymotrypsin and pepsin	Catalyzes the hydrolysis of peptide bonds of proteins in the small intestine	45
Trypsin	Anti-inflammatory agent, wound cleanser	44, 46, 47
Streptokinase	Anti-inflammatory agent, dissolution of blood clots in myocardial infarction	45
Hyaluronidase	Hydrolyses polyhyaluronic acid, a relatively impermeable polymer found between human cells; administered to increase diffusion of coinjected compounds, e.g., antibiotics, adrenaline, heparin, and local anesthetic in surgery and dentistry.	48–51
Heparinase	Removal of heparin after surgery. Production of heparin oligosaccharides (wound healing and tumor netastasis properties).	52, 53
Urokinase	Prevention and removal of blood clots	54–56
Streptodornase	Anti-inflammatory agent	57
Tissue plasminogen activator (TPA)	Dissolution of blood clots	58
Tyrosinase	Enzyme essential for the production of cellular pigments	20, 59, 60
Bilirubin oxidase	Treatment of neonatal jaundice	61
Penicillinase	Removal of allergenic form of penicillin from allergic individuals	16
Alkaline phosphatase	Treatment of hypophosphatasia	15

involves the isolation of the enzyme within a microcapsule, fiber, or gel, which will protect the enzyme from proteolysis and avoid the undesirable immunological response (please see Section 17.3.2.2 of this chapter).

The pharmacological properties of enzymes have been employed to replace enzymes that are missing or defective as a consequence of an inherited disease or malfunction of an organ where they are normally synthesized or to accomplish a certain biological effect that is dependent on the catalytic activity of the enzyme. Therefore, depending on the treatment, the administration of enzymes as therapeutic agents can be subdivided into two categories[9,11]: (1) the topical application of an enzyme as an extracellular agent and (2) the intracellular applications of enzymes to treat metabolic deficiency and related disease. The main areas where enzyme therapy has been applied are the degradation of necrotic tissue by the use of proteolytic enzymes, removal of toxic compounds from the blood, treatment of genetic deficiency diseases and cancer, and treatment of pancreatic insufficiency.[11] In Table 17.2 are given some examples of areas where enzyme therapy may be used.

Enzymes may be administered either intra- or extracorporeally, depending on the objective. If the enzyme is to be used for the removal or transformation of a substance present in the blood (e.g., toxic metabolite or a blood clot), then it is only necessary for the enzyme to be present in the blood and not necessary for the enzyme to enter the intracellular compartments. This type of application may be either intra- or extracorporeal using a bypass as in kidney dialysis. These systems

will be described in more detail in Section 17.3.2.3 of this chapter. For intracellular therapy, it is necessary for the enzyme to be taken up by the appropriate target cells.

Although the attempts made with enzyme therapy in clinical trials have so far had limited success, it is reasonable to assume that the delivery of enzymes (discussed in Section 17.3.2.2 of this chapter) would constitute a feasible approach for the treatment of certain diseases in the near future.

17.3 ENZYME IMMOBILIZATION TECHNOLOGY

The term *immobilized enzyme* was adopted in 1971[62] at the first Enzyme Engineering Conference. It describes enzymes physically confined at or localized in a certain region of space with retention of catalytic activity and which can be used repeatedly and continuously.[63] The immobilization of biocatalysts (not only enzymes but also other bioactive molecules such as growth factors and hormones, cellular organelles, microbial cells, and plant and animal cells) is attracting worldwide attention in biotechnology applications. In general, immobilized biocatalysts are more stable and easier to handle compared with their free counterparts.[64] At present, applications of immobilized biocatalysts include the production of useful compounds by stereospecific or regiospecific bioconversion, the production of energy by biological processes, the selective treatment of specific pollutants to solve environmental problems, continuous analyses of compounds with a high sensitivity and specificity, and medical uses such as new types of drugs for enzyme therapy or artificial organs.[14] Immobilized enzymes are already being used in medical applications for clinical diagnosis and also for intra- and extracorporeal enzyme therapy.[62] Applications in clinical analysis are mainly related to biosensors, which have been used to detect the presence of various organic compounds for many years. For example, glucose oxidase and catalase have been used to measure blood glucose concentration, and cholesterol oxidase and cholesterol esterase to determine cholesterol levels.[31] In addition, enzymes can be immobilized on different prosthetic devices or used extracorporeally (e.g., artificial heart, artificial lung, artificial kidney, equipment for hemodialysis and specific blood purification) as surface modifiers in order to increase the biocompatibility of these devices and to prevent blood clotting.[9]

17.3.1 Methods for Immobilizing Enzymes in Polymeric Carriers

Various methods have been developed[10,65] for the immobilization of biocatalysts, which are being used extensively today. A wide range of support materials has also been employed for enzyme immobilization. The support type can be classified according to their chemical composition, such as organic or inorganic supports, and the former can be further classified into natural or synthetic matrices.[66] Immobilization techniques can be divided into different categories: physical,[67] chemical,[68] enzymatic,[69] and genetic engineering methods.[53]

17.3.1.1 Adsorption

The adsorption of an enzyme onto a support or film material is the simplest method of obtaining an immobilized enzyme. Basically, the enzyme is attached to the support material by noncovalent linkages and does not require any preactivation step of the support. The interactions formed between the enzyme and the support material will be dependent on the existing surface chemistry of the support and on the type of amino acids exposed at the surface of the enzyme molecule. Enzyme immobilization by adsorption involves, normally, weak interactions between the support and the enzyme such as ionic or hydrophobic interactions, hydrogen bonding, and van der Waals forces (see Figure 17.1).[70,71]

Most of the support materials available have sufficient surface-charge properties suitable for immobilization by adsorption. They include inorganic carriers[62] (ceramic, alumina, activated carbon, kaolinite, bentonite, porous glass), organic synthetic carriers[72] (nylon, polystyrene), and natural

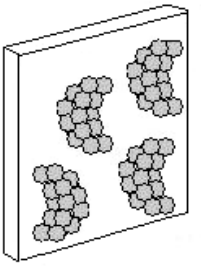

FIGURE 17.1 Biocatalysts bound to a carrier by adsorption.

organic carriers[1] (chitosan, dextran, gelatin, cellulose, starch). The method consists of simply mixing an aqueous solution of enzyme with the support material for a period of time, after which the excess enzyme is washed away from the immobilized enzyme on the support. The procedure requires strict control of the pH and ionic strength, because these can alter the charges of the enzyme and the support and therefore affect the level of adsorption. A simple shift in pH can cancel ionic interactions and promote the release of the enzyme from the support. The main advantages of adsorption are the method simplicity, the little effect on the conformation/activity of the biocatalyst, and the possibility of regenerating inactive enzyme by addition of fresh enzyme. The main disadvantage is the desorption of the biocatalyst from the support due to the weak interactions established. The enzyme desorption can easily occur by changes in the environment medium such as pH, temperature, solvent, and ionic strength or in the case of extended reactions.[73]

17.3.1.2 Ionic Binding

Immobilization via ionic binding is based, mainly, on ionic binding of enzyme molecules or active molecule to solid supports containing ionic charges. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depends on the nature of the carrier. Figure 17.2 shows how the enzyme is bound to the carrier. In some cases, physical adsorption may also take place. The main difference between ionic binding and physical adsorption is the strength of the interaction, which is much stronger for ionic binding, although less strong than covalent binding. The preparation of immobilized enzymes using ionic binding is based on the same procedure as described for physical adsorption.[74,75] The ionic nature of the binding forces between the enzyme and the support also depends on pH variations, support charge, enzyme concentrations, and temperature. The supports used for ionic binding may be based on polysaccharide derivatives[64] (e.g., diethylaminoethylcellulose, dextran, chitosan, carboxymethylcellulose), synthetic polymers[10] (e.g.,

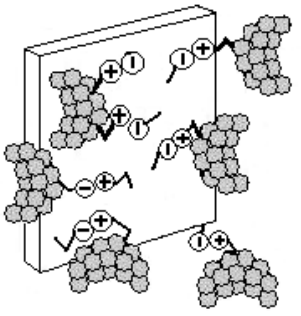

FIGURE 17.2 Biocatalysts bound to a carrier by ionic binding.

polystyrene derivatives, polyethylene vinylalcohol), and inorganic materials[62] (e.g., ambertite, alumina, silicates, bentonite, sepiolite, silica gel, etc.). The immobilization by ionic binding has the advantage that changes in the enzyme conformation only occur in a small extent, resulting in immobilized enzymes with high enzymatic activities. The main disadvantage is the possible interference of other ions, and special attention should be paid in maintaining the correct ionic strength and pH conditions in order to prevent their easy detachment.[10]

17.3.1.3 Covalent Binding by Chemical Coupling

The covalent binding method is based on the binding of enzymes, or other active molecules, to a support or matrix by means of covalent bonds.[10] The bond is normally formed between a functional group present on the support surface and amino acid residues on the surface of the enzyme. Those which are most often involved in covalent binding are[65] the amino (NH_2) group of lysine or arginine, carboxyl (CO_2H) group of aspartic acid or glutamic acid, hydroxyl (OH) group of serine or threonine, and sulphydryl (SH) group of cysteine.[76,77] There are many reaction procedures for joining an enzyme to a material with a covalent bond (diazotation, amino bond, Schiff's base formation, amidation reactions, thiol-disulfide, peptide bond, and alkylation reactions). The connection between the support and the biocatalyst can be achieved either by direct linkage between the components or via an intercalated link of different length, the so-called spacer or harm. The advantage of using a spacer molecule is that it gives a greater degree of mobility to the coupled biocatalysts so that its activity can, under certain circumstances, be higher than if it is bound directly to the support (see Figure 17.3). It is important to choose a method that will not involve the reaction with the amino acids present in the active site, since this could inactivate the enzyme. Basically, two steps are involved in the covalent binding of enzymes to a support material. First, functional groups on the support material are activated by specific reagents (e.g., cyanogen bromide, carbodiimide, aminoalkylethoxysilane, isothiocyanate, and epichlorohydrin, etc.). A large range of support materials is available for covalent binding, and this extensive range reflects the fact that no ideal matrix exists. Therefore, the advantages and disadvantages of a given matrix must be taken into account when considering the appropriate procedure for a given enzyme immobilization. Immobilization of enzymes through covalent attachment has also been demonstrated[73] to induce higher resistance to temperature, denaturants, and organic solvents in several cases. The extent of these improvements may depend on other conditions of the system, e.g., the nature of the enzyme, type of support, and the method of immobilization. Many factors may influence the selection of the support, and some of the more important are its cost and availability, the binding capacity (amount of enzyme bound per given weight of matrix), hydrophilicity (the ability to incorporate water into the matrix and stability of matrix), structural rigidity, and durability during applications. Natural polymers, which are very hydrophilic, are popular support materials for enzyme immobilization since the residues in these

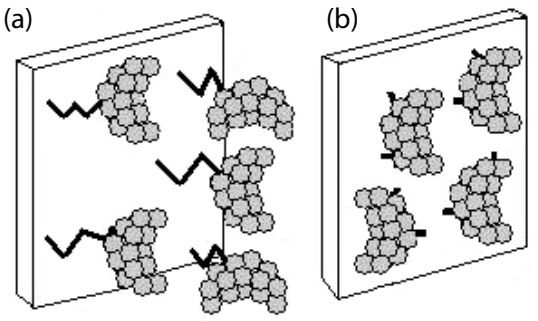

FIGURE 17.3 Covalent bond between the biocatalysts and a carrier with (a) and without spacer (b).

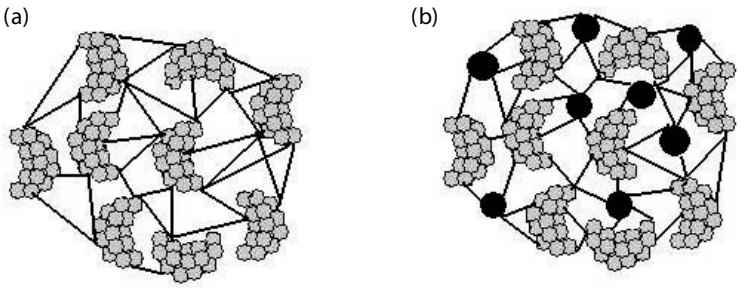

FIGURE 17.4 Biocatalysts immobilized by means of crosslinking (a) and co-crosslinking with inert molecules incorporated (b).

polymers contain hydroxyl groups, which are ideal functional groups for participating in covalent bonds. A frequently encountered disadvantage of immobilization by covalent binding is that it places great stress on the enzyme. The necessary harshness of the immobilization procedure nearly always leads to considerable changes in conformation and a resultant loss of catalytic activity.[72,78,79]

17.3.1.4 Crosslinking

The crosslinking method is based on the formation of covalent bonds between the enzyme or active molecules, by means of bi- or multifunctional reagents.[80,81] The individual biocatalytic units (enzymes, organelles, whole cells) are joined to one another with the help of bi- or multifunctional reagents (e.g., glutardialdehyde, glutaraldehyde, glyoxal, diisocyanates, hexamethylene diisocyanate, toluene diisocyanate, etc.). Enzyme crosslinking involves normally the amino groups of the lysine but, in occasional cases, the sulfhydryl groups of cysteine, phenolic OH groups of tyrosine, or the imidazol group of histidine can also be used for binding. Figure 17.4 shows how the biocatalysts can be linked by a simple crosslinking process (Figure 17.4a) and also by co-crosslinking, in which inert molecules are incorporated in the high-polymer network in order to improve the mechanical and enzymatic immobilized preparation (Figure 17.4b).

The advantages and disadvantages of a given matrix must be taken into account when considering the appropriate procedure for a given enzyme immobilization. One advantage is the simplicity of the process. The main disadvantages are the fragility of the particles produced in some cases and diffusion limitations. Since crosslinking and co-crosslinking usually involve covalent bonds, immobilized biocatalysts in this way frequently undergo changes in the conformation with a resultant loss of activity. The isomerization of glucose process is a very important example of the industrial application using biocatalysts crosslinked with glutaraldehyde. Some of the immobilized preparations used in these large-scale processes are produced simply by glutaraldehyde treatment of bacterial cell masses that have formed fine particles.[10]

17.3.1.5 Entrapment and Encapsulation

The entrapment method for immobilization consists of the physical trapping of the active components into a film, gel, fiber, coating, or microencapsulation[73] (see Figure 17.5). This method can be achieved by mixing an enzyme or active molecule with a polymer and then crosslinking the polymer to form a lattice structure that traps the enzyme. Microencapsulated enzymes are formed by enclosing enzymes solution within spherical semipermeable polymer membranes with controlled porosity.

While the encapsulation of dyes, drugs, and other chemicals has been known for some time, it was not until the mid-1960s that such a method was first applied to enzymes. Since that first report, a number of other enzymes have been successfully immobilized via microencapsulation,

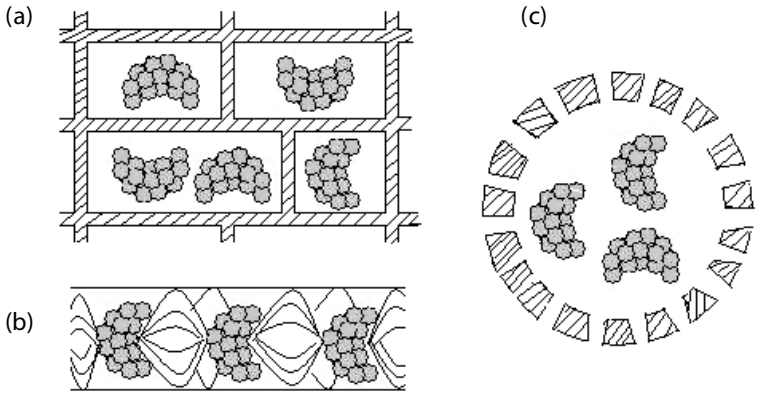

FIGURE 17.5 Enzyme encapsulation in a matrix (a), fiber (b), or capsule (c).

using a number of different materials and methods to prepare the microcapsules. The advantages of this immobilization method are the extremely large surface area between the substrate and the enzyme, within a relatively small volume, and the real possibility of simultaneous immobilization. The major disadvantages of this method include the occasional inactivation of enzyme during microencapsulation and the high enzyme concentration required. In addition, to retain the enzyme, the pore size needs to be very low and these systems tend to be very diffusion limited.[72]

17.3.1.6 Protein Fusion to Affinity Ligands and Enzymatic Conjugation

As described before, there are many methods for protein immobilization, but some of them require chemical modification of the matrix, which may result in material degradation, especially when biodegradable polymers are used. In addition, these modifications, necessary to attach the enzyme to the matrix, often result in the loss of enzyme activity as well as the inclusion of toxic organic compounds, which have to be removed before the system can be used in biomedical applications. In this type of application, the efficacy of immobilized biomolecules for stimulating specific cell responses (e.g., proliferation or differentiation) depends on the mode by which these modulators are presented to the target cells. In these cases, it is important to ensure the correct orientation and full bioactivity of the molecules when they are immobilized. Covalent binding by chemical coupling, again, might hinder ligand–receptor interaction or prevent receptor dimerization and capping on target cells.

In nature, there are certain protein molecules, such as lectins,[14,82] avidin,[83] immunoglobulin G (IgG) binding domains of protein G and protein A,[84,85] and carbohydrate binding modules[86,87] (present in many polysaccharide-degrading enzymes), that bind with high affinity and specifically to certain molecules or solid surfaces. These binding domains may be used as affinity tags for immobilizing proteins to affinity adsorbents. In this technique, DNA encoding a polypeptide affinity tag is fused to the gene of interest, and the expression of the gene results in a fusion protein. Such a fusion protein could be immobilized by the specific binding of the affinity tag to an affinity adsorbent (Figure 17.6a). With this method, the conformational changes in the protein upon immobilization are minimal, and the immobilized biomolecule could retain high activity. In addition, since fusion proteins are specifically immobilized on the support materials, these methods normally allow the immobilization of high densities of ligands and can also simplify the immobilization procedure.

The use of genetic engineering techniques to construct chimeric proteins, containing a functional domain displaying bioactivity together with an affinity domain, has proven to be a very useful approach for immobilizing biomolecules on solid materials. Several proteins, including

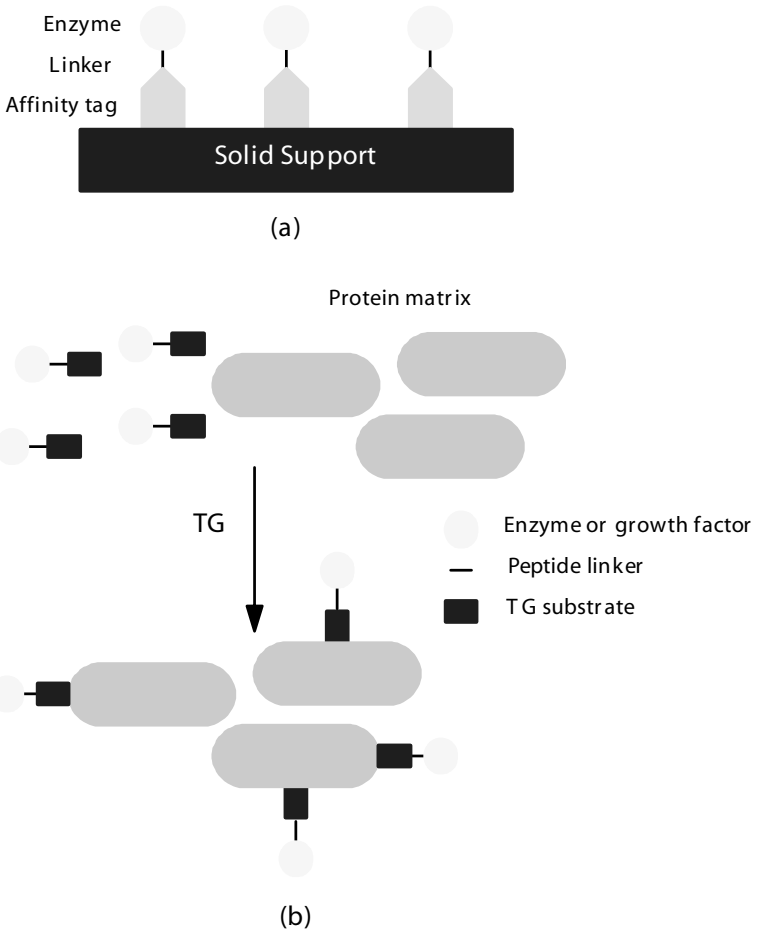

FIGURE 17.6 Enzyme immobilization to solid matrices via protein fusion to an affinity ligand (a) and enzymatic conjugation catalyzed by transglutaminase (b).

enzymes,[53,88,89] antibodies,[90,91] cytokines,[92] and streptavidin,[93] have been immobilized on the surface of cellulosic matrices by fusing genetically these proteins to cellulose-binding domains.

By using protein fusion techniques, bifunctional proteins can be prepared without changing their activity and binding properties.[53,87,89] This approach has found various applications in biotechnology, diagnostics, and medicine for the purification and immobilization of biologically active proteins. It can be used, for instance, to promote the attachment of several mammalian cells to different surfaces by fusing a variety of peptides, growth factors, and cytokines to a specific binding domain, and this might be useful for the activation and growth of progenitor cells in culture.

The binding affinity between streptavidin and biotin is among the strongest noncovalent bonds known to exist ($K_D = 10^{-15}$ M).[83] Therefore, the high affinity coupling of the biotin–avidin system has been used to immobilize different biomolecules on the surface of biomaterials and biosensors.[94,95] It consists of using avidin as a bridge between the biotinylated surface and biotinylated short ligand molecules.

The immobilization of biomolecules on protein matrices may also be achieved via an enzymatic reaction catalyzed by transglutaminase (TG) enzyme. TG catalyzes the acyl transfer reaction between the γ-carboximine group of a peptide-bound glutaminyl residue and a primary amino group of various protein substrates. The result of this reaction is the formation of an irreversible crosslinked, insoluble supramolecular structure.[96] In addition, TG can be used to bind glutamine-

containing peptides or polypeptides to NH_2 surfaces,[69] and this methodology is schematically represented in Figure 17.6b. This approach was used by Sakiyama et al.[97,98] to incorporate heparin-binding peptides and to design a growth factor delivery system. In the latter work, they developed a growth factor fusion protein, containing a bi-domain peptide consisting of a β-nerve growth factor (β-NGF) and an exogenous factor XIIIa (substrate for enzymatic crosslinking). The fusion protein was covalently immobilized within a three-dimensional cell in-growth matrix based on fibrin using the transglutaminase activity of factor XIIIa.

17.3.2 Enzyme Immobilization in Biomedical Applications

Biomaterials can be combined with biomolecules, such as enzymes and growth factors, to yield biologically functional systems. There is a wide and diverse range of materials and methods available for enzyme immobilization on or within the biomaterial. The methods for immobilizing enzymes and other biomolecules are the same as described in Section 17.3.1 of this chapter, but the choice of the method depends largely on the final application. Furthermore, the criteria for selecting the immobilization methods should also take into account that the immobilized enzyme should retain an acceptable level of activity over a certain period of time in terms of economic or clinical aspects.

The methods used for the administration of immobilized enzymes may be divided into two principal groups[9]: immobilized enzymes that are intended for prolonged circulation and enzymes that must be necessarily present in different tissues and organs of the body. In the second case, the immobilized enzyme is intended for local deposition during the treatment of discrete lesions (e.g., thrombi, tumors, atherosclerotic injuries) or of the individual organs.[9]

Table 17.3 describes some examples of immobilized enzymes in various biomaterial supports for different biomedical applications.

17.3.2.1 Biologically Functional Surfaces

Biomaterials, especially when used in tissue engineering applications, must have the capacity to induce tissue regeneration/repair in order to achieve a more rapid recovery of the defect. At present, the existing scaffolds are not satisfactory in achieving rapid and full recovery of the defect. The attachment of cells to biomaterials, and their subsequent spreading, are mediated by extracellular matrix (ECM) glycoproteins such as fibronectin, collagen, etc. ECM glycoproteins contain short sequences with cell attachment properties, which interact with the integrin family of cell surface receptors. The peptide sequence Arg-Gly-Asp (RGD) has been identified as being capable of interacting with cell surface receptors. Thus, the immobilization of biologically active molecules on the surface of biomaterials for presenting effectors to target cells or to induce a particular effect is of great interest, since the immobilization of active agents presents the advantage of providing a continuous and localized stimulus for cell proliferation. Unlike nonimmobilized active agents, which are often consumed by cells, immobilized biomolecules remain bound to a substrate that is not consumed by cells and thus remain available to stimulate growth of additional cells. This is particularly useful in perfusion cultures in which growth medium is continuously added and removed to allow long-term cell proliferation.

Biodegradable polymers have been used as scaffolding materials for various tissue engineering applications because they can provide the support on which cells and tissues can adhere, but they can also guide and regulate the proliferation and activities of the adhered cells. However, the intrinsic hydrophobic property of some of these polymers[108] restricts their applications as cell colonizing materials. Many methods have been used to modify the properties of polymer surface, such as plasma treatment-induced grafting polymerization, ozone oxidation, and immobilization of enzymes, and special biologically active agents have been used to introduce reactive groups onto polymeric surfaces.[109]

TABLE 17.3
Examples of Immobilized Enzymes in Various Biomaterials for Different Biomedical Applications

Enzyme	Biomaterial (Carrier)	Immobilization Method	Application	Ref.
Alkaline phosphatase	pHEMA[a]	Entrapment	Induction of bone and cartilage mineralization	99
	Glassy carbon	Protein fusion to affinity tag	Biosensor/enzyme-linked immunoassays	95
α-amylase	pHEMA[a]	Covalent binding	Fixed-bed reactor	100
	Starch polymeric blend	Encapsulation	Tailor the degradation rate of starch-based biomaterials	101
β-galactosidase	Gelatin	Encapsulation	Not specified	102
Glucose oxidase	Proteins	Enzymatic conjugation	Biosensor	69
α-chymotrypsin	Cellophane	Plasma modification/ covalent binding	Not specified	103
Acetylcholinesterase	pHEMA[a]	Entrapment	Biosensor	104
Choline oxidase		Covalent binding Ionic interactions		
Papain	Polyethylene	Plasma modification	Not specified	105
	Glass surfaces	Covalent binding		
Lysozyme	Gelatin and succinylated gelatin	Adsorption Ionic interactions	Reduction of prosthetic valve endocarditis	106
	Poly(ethylene glycol)–poly(aspartic acid) copolymer	Entrapment/ionic binding	Delivery of lysozyme as a lytic enzyme	28
Heparinase	Sepharose	Covalent binding	Bioreactor for extracorporeal elimination of heparin from blood	52
Phospholipase A_2	Agarose	Not mentioned	Bioreactor for treatment of hypercholesterolemia (reduce plasma cholesterol)	107
Bilirubin oxidase	Agarose	Covalent binding	Bioreactor for removal of bilirubin from blood	61
Prolidase	PLGA[b] microspheres	Encapsulation	Enzyme replacement therapy	25

[a] Poly(2-hydroxy ethyl methacrylate).
[b] Poly(*D,L*-lactide-co-glycolide).

Various strategies have been developed to incorporate bioactive agents on the surface of biomaterials for controlling cell and tissue responses. The immobilization process can be involved, enriching surfaces for enhancing the cellular adhesion. Biomolecules such as enzymes, antibodies, antigens, peptides, or drugs have been immobilized on or within polymeric systems. An example of an adhesive protein is fibronectin, which is able to promote cellular adhesion through binding to integrin receptors, and this interaction has also been shown to play a role in cell growth, differentiation, and overall regulation of cell function.[77,110] Hern and Hubbell[111] showed that the incorporation of the adhesion peptide RGD into a nonadhesive hydrogel proved to be useful for tissue resurfacing. There are numerous other adhesion peptides for targeting particularly desirable cell types and to modulate biological responses.

Urokinase has been widely used for the clinical treatment of thrombogenetic disease and hemorrhoidal disease. Artificial organ materials, on which urokinase was immobilized for its

fibrinolytic activity, have been developed for blood-compatible materials. For example, Liu et al.[55] immobilized urokinase by encapsulation in poly(2-hydroxyethyl methacrylate) and König et al.[56] introduced urokinase on the surface of the polytetrafluoroethylene using plasma modification technique by covalent bond. Another example of immobilized urokinase application was reported by Kato and coworkers,[54] who had used urokinase immobilized in a Teflon catheter for treatment of thrombosis.

Most of the studies found in the literature, regarding the surface functionalization of biomaterials with biological molecules, include the incorporation of adhesion and differentiation factors. The same approach may be used to incorporate specific enzymes able to regulate a number of cell functions. For instance, it is known that mitogen activated protein (MAP) kinase, upon activation by dual phosphorylation at threonine and tyrosine residues, is able to activate downstream targets that have been implicated in controlling gene expression, cell differentiation, and proliferation.[112] This enzyme may be immobilized on the surface of biomaterials to control cell response, but other possibilities using different enzymes remain unexplored.

17.3.2.2 Enzyme Delivery

Although purified enzymes are now available for some enzyme deficiency diseases (see Table 17.1), there are many problems in delivering the enzyme to the required site under such conditions that it will remain stable and active for a reasonable time. Normally, quite large amounts of enzyme are necessary with high level of purity and in a nonimmunogenic form.[9,14,113,114] In addition, many enzymes when administered are inactivated or degraded fairly rapidly. The delivery of therapeutic molecules requires, therefore, efficient strategies to have a precise control on their release profile according to specific locations. It might be possible to control the release of such molecules by creating delivery systems sensitive to changes in pH, temperature, or salt concentration or to the feedback provided by cells. The concept of enzyme-activated drugs in therapy is scientifically, as well as clinically, attractive, as it allows the chemist and enzymologist full intellectual rein in designing interlinked systems.[114] As therapeutic drugs, enzymes possess several attributes such as high specificity toward substrate, high solubility for preparing liquid formulations, and optimum activity under physiological conditions.[115] The administration of enzymes, in cases of enzyme deficiency and inborn errors of metabolism and in the treatment of certain types of cancer, appears to offer a successful form of therapy. Cancer therapy based on the delivery of enzymes to tumor sites has advanced in several directions since antibody-directed enzyme/prodrug therapy was first described.[116] Nanospheres, nanocapsules, liposomes, micelles, and other nanoparticulates are frequently referred to as carriers for delivery of therapeutic and diagnostics agents.[113]

Asparagine is an essential amino acid for certain types of leukemias that lack asparagines synthetase activity. The activity of *L*-asparaginase is to degrade asparagine into aspartate and ammonia. Therefore, asparaginase has been of interest to biochemists and clinicians as a possible cancer therapeutic agent. Some success has been achieved in administering asparaginase in capsules made of nylon and polyurea to mice and rats. Although asparaginase has been found to be effective in the treatment of some patients,[117] it may have several serious side effects.[41] Relatively high concentrations of the enzyme are needed for it to be clinically effective. These levels cause a wide range of toxic effects on several organs including the liver, pancreas, kidneys, and brain. The enzyme may also be recognized as foreign by the body and potentially severe immunogenic responses will be stimulated, resulting in hypersensitivity reactions. To overcome these problems, immobilized enzyme derivatives have been prepared on various supports for extracorporeal treatment (please see Section 17.3.2.3 of this chapter).

Enzymes may be also used in cancer therapy as prodrug activators. This therapy consists basically in using a drug that has been chemically modified so that it remains inactive until specifically activated by an enzyme at the target site.[11]

Another example of an anticancer enzyme is hyaluronidase.[50] Hyaluronidase is a globular enzyme of endoglycosidase action, which can depolymerize hyaluronic acid in the organism, decreasing its viscosity and increasing tissue permeability. Hyaluronidase has been utilized extensively as an adjunct in anticancer chemotherapy regimens, suggesting that hyaluronidase has intrinsic anticancer properties against tumor growth.[51] *In vitro* studies in tissue culture with tumor spheroids and *in vivo* tests using animal models demonstrated the beneficial effect of hyaluronidase for the penetration of drugs into tumor tissue. Later, in a prospective clinical trial, hyaluronidase significantly improved the outcomes of patients with bladder carcinoma if the enzyme was administrated topically together with mitomycin C.[49,50] Hyaluronidase is also used for local application (subcutaneous injections) during treatment of joint disease, in dermatology, and in ophthalmology.[48] Hyaluronidase had been used in ophthalmology with the aim of formation of a thinner scar and to prevent necrosis after paravataes with zytostatics.

Trypsin has also been used to remove dead tissue from wounds, burns, and ulcers to speed the growth of new tissue and skin grafts, as well as to inhibit the growth of some contaminant organisms. The inappropriate activation of trypsinogen within the pancreas leads to development of pancreatitis.[47] Once trypsin is activated, it is capable of activating many other digestive pro-enzymes. These activated pancreatic enzymes further enhance the auto-digestion of the pancreas. Many materials, such as nylon, polysulfone, glycidyl methacrylate, chitosan, cellulose, and cellulose derivatives, have been used for trypsin immobilization.[44,46,47]

Enzyme therapy has also been tested for pancreatic insufficiency and cystic fibrosis. Pancreatic insufficiency can be alleviated by administrating orally enteric-coated microspheres containing lipase, amylase, and proteases. A special polymer coating protects the enzymes at low pH, such as in the stomach, and then releases them in the intestine at physiological pH.[11,14]

Lysozyme is a good example of an enzyme that catalyzes chemical reactions in the cell. Lysozyme acts to kill bacteria by cleaving the covalent bond between the alternating polysaccharides that compose peptidoglycan in bacterial cell walls.[27] The human salivary defense proteins and lysozyme are known to exert a wide antimicrobial activity against a number of bacterial, viral, and fungal pathogens *in vitro*. Therefore, these proteins, alone or in combinations, have been incorporated as preservatives in foods and pharmaceuticals as well as in oral health care products to restore saliva's own antimicrobial capacity in patients with dry mouth. These antimicrobials used in oral health care products, such as dentifrices, mouth rinses, moisturizing gels, and chewing gums, have been purified from bovine colostrum. Other studies had been reported with lysozyme bound to chitosan, silica gel by means of physical adsorption, crosslinking to a polystyrene divinylbenzene matrix by the formation of ionic bindings, and by covalent attachment to nonporous glass beads.[29] Harada and Kataoka[28] described lysozyme immobilized into poly(ethylene glycol)–poly(aspartic acid) micelle. Lysozyme was selected as a model protein to incorporate into the micelle because it has a high isoelectric point (pI = 11), is positively charged over a wide range of pH, and has practical usage in drug delivery application as a lytic enzyme. Chen and Chen[118] prepared immobilized lysozyme by carbodiimide method to form amide bonds with an enteric coating polymer (hydroxypropyl methyl-cellulose acetate succinate [AS-L]) as the carrier, which shows reversibly soluble-insoluble characteristics with pH changes.

The glucose-6-phosphate dehydrogenase enzyme catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate while concomitantly reducing the oxidized form of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to nicotinamide adenine dinucleotide phosphate (NADPH). NADPH, a required cofactor in many biosynthetic reactions, maintains glutathione in its reduced form. Reduced glutathione acts as a scavenger for dangerous oxidative metabolites in the cell. With the help of the enzyme glutathione peroxidase, reduced glutathione also converts harmful hydrogen peroxide to water. Red blood cells rely heavily on glucose-6-phosphate dehydrogenase activity because it is the only source of NADPH that protects the cells against oxidative stresses. People deficient in glucose-6-phosphate dehydrogenase are not prescribed, therefore, with oxidative drugs because their red blood cells undergo rapid hemolysis under this stress.[36] In

Greece, glucose-6-phosphate dehydrogenase deficiency is the main cause of severe neonatal jaundice. The deficiency of this enzyme affects all races; the highest prevalence is among persons of African, Asian, or Mediterranean descent.[37] Study of immobilized glucose-6-phosphate dehydrogenase has been reported. Kotorman et al.[38] immobilized glucose-6-phosphate dehydrogenase from yeast on polyacrylamide beads possessing carboxylic functional groups activated by a water-soluble carbodiimide. They verified highest operational stability of immobilized glucose-6-phosphate dehydrogenase.

In relation to the use of immobilized enzymes with therapeutic purposes, catalase is one of the most interesting because it is employed to accelerate healing as well as to correct hereditary deficiencies and, in combination with hydrogen peroxide, as an antiseptic against anaerobes.[33,34] The catalase enzyme has the ability to decompose hydrogen peroxide into oxygen and water, playing a central role in controlling the hydrogen peroxide concentration in human cells. More than 98% of blood catalase is localized in erythtocytes.[119] These cells, with their high catalase level, provide a general protection against the toxic concentration of this small hydrogen peroxide molecule. The deficiency of catalase could cause acatalasemia.[35,119] Several methods have been developed for the immobilization of catalase.[1,74,120] Immobilization is often accompanied by changes in the enzymatic activity, optimum pH, affinity to the substrate, and stability. The extent of these changes depends on the enzyme, carrier support, and the immobilization conditions.[121] The shift in the optimum pH, from acidic or alkaline to neutral pHs, may be useful for biomedical applications since it will allow the use of some enzymes (more active at low or high pHs) under more physiological conditions.

The approach developed by Sakiyama-Elbert et al.,[98] consisting in a cell-triggered growth factor delivery system, may also be used for the release of other important therapeutic molecules.

17.3.2.3 Bioreactors for Extracorporeal Enzyme Therapy

Extracorporeal shunts have been proposed[72,122] for the treatment of several clinical conditions. The most likely applications for enzymatic treatment are the removal of urea during kidney failure, removal of toxins (e.g., paracetamol) during liver failure, or the reduction of key metabolites from the circulation to treat cancer.

Urease is one of the most important enzymes in biomedical applications. Urease is an enzyme that catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. Urea is one of the main metabolic end products, and the removal of its excess has been a major problem for patients suffering from renal failure. Hence, its immobilization by entrapment has been investigated by many workers for applications in biosensors and as artificial kidneys. The most attention has been given to the development of enzyme reactors, where the urea would be removed and the dialysis fluid prepared for further use.[30,31] The use of this enzyme is often limited due to its high cost, availability in small amounts, instability, and the limited possibility of feasible recovery of these biocatalysts from a reaction mixture. Numerous synthetic and natural polymeric supports have been used for urease immobilization, and their uses in medical and technical fields are well reported. The covalent bond of urease in different supports has been reported in many studies. Some commonly used supports are chitosan-poly(glycidil methacrylate), carboxymethylcellulose, polyurethane, sepharose-2B, polyacrylamide, ion exchange resins, copolymers of polyglycidylmethacrylate, calcium alginate beads, poly(vinyl alcohol) (PVA), hydroxyapatite, 2-dimethylaminoethylmethacrylate, poly(ethylene glycol dimethacrylate/2-hydroxy ethylene methacrylate) microbeads, poly(caprolactone)/starch, and poly(orthoesters).[32,109,123–125]

As mentioned before in Section 17.3.2.2 of this chapter, *L*-asparaginase has been used for treating leukemias and disseminating cancers that require asparagines for growth, but this treatment presents several serious side effects. To overcome these problems, immobilized enzyme derivatives have been prepared on various supports for extracorporeal treatment. Blood can be passed over the immobilized enzyme, thus depleting the asparagine supply needed by the cancer cells. The enzyme does not come into direct contact with the organs to which it is toxic, and hypersensitivity

reactions do not occur. With this type of treatment, however, the blood plasma must be first separated from the cells to minimize cell damage and then passed through a separate column containing the immobilized enzyme. This process requires that the blood remain outside the body for relatively long periods of time, resulting in the denaturation and depletion of many plasma proteins.[41,42] Some techniques have been developed to minimize this problem, such as the use of a porous hollow-fiber plasmapheresis device. With this system, the plasma can be separated from the whole blood and contact with immobilized enzyme in one passage, thus minimizing its time outside the body and then reducing the damage to the plasma proteins. Adsorption techniques have been used to immobilize asparaginase onto hollow fibers after first coating the fibers with albumin and then crosslinking the enzyme with glutaraldehyde.[41,126] Maciel and Minim[43] also reported that the use of *L*-asparaginase covalently attached to nylon tubing may constitute a useful system to be used in clinical applications.

Bilirubin oxidase is also an example of enzyme used in extracorporeal applications.[61] All human newborns accumulate bilirubin to levels greater that those in adults, and 20% accumulate enough to stain their skin, resulting in jaundice. Bilirubin binds to cellular and mitochondrial membranes, causing cell death in a variety of tissues. Clinically, bilirubin toxicity may lead to mental retardation, cerebral palsy, deafness, seizures, or death. The most common treatments for jaundiced infants are phototherapy and exchange transfusion. This technique presents serious problems such as hypoglycemia, hypocalcemia, acidosis, transmission of infectious, etc. Lavin et al.[61] reported the use of a highly specific enzyme to remove bilirubin from the bloodstream using a small reactor (extracorporeal circuit) containing bilirubin oxidase covalently immobilized in agarose beads. These researchers obtained good results for the removal of the bilirubin in humans and in genetically jaundiced rats.

Heparinase, an enzyme that degrades heparin into small polysaccharides, has also been immobilized into an extracorporeal device (artificial kidney bioreactor) to eliminate the anticoagulant properties of heparin (used to prevent clotting in the device) before the blood returns to the patient.[52]

17.3.2.4 Diagnostic Assays and Biosensors

Isolated or combined enzymes are being used in medicine as useful tools for clinical analysis. About 50 different enzymes are used in different aspects of clinical diagnoses, and for most of these, much higher levels of purity are required than for most industrial enzymes. Two of the major enzymes used are peroxidase from horseradish and alkaline phosphatase from beef intestinal mucosa, both being required for immunoassays. The enzymes may be used in test strips, ELISA, biosensors, and autoanalyzers.[11]

The serum uric acid concentration is an important index for clinical diagnosis of gout, leukemia, toxemia of pregnancy, and severe renal impairment.[127] A number of enzymes are assayed in serum and urine for diagnostic purposes; the more frequently used ones are discussed below.

Alkaline phosphatases (ALP) are a group of enzymes found primarily in the liver (isoenzyme ALP-1) and bone (isoenzyme ALP-2). There are also small amounts produced by cells lining the intestines (isoenzyme ALP-3), the placenta, and the kidney (in the proximal convoluted tubules). What is measured in the blood is the total amount of alkaline phosphatase released from these tissues into the blood. As the name implies, this enzyme works best at an alkaline pH (pH 10), and thus the enzyme itself is inactive in the blood. Alkaline phosphatase acts by splitting off phosphorus (an acidic mineral), creating an alkaline pH. The primary importance of measuring alkaline phosphatase is to check the possibility of bone or liver diseases.[128]

Another application of immobilized enzymes is the development of improved sensing devices.[9] Because of their high specificity for given substances, enzymes and monoclonal antibodies are particularly suitable for use as sensors.[129] The membrane-covered electrode described by Clark in 1959 is the dominating sensor for the measurement of dissolved oxygen.[130] Numerous modifications of the original concept have been developed. For instance, biosensors using enzymes have been used to detect the presence of various organic compounds, and recent developments have proven

to be both rapid and highly selective. They have been used in important applications such as in clinical laboratories, fermentation processes, and pollution monitoring.[131] Most of them have used a free or immobilized enzyme and an ion-sensitive electrode that measures indirectly (e.g., by temperature or color changes produced by an enzymatic reaction) the presence of a product whose formation is catalyzed by the enzyme. The biosensors usually have immobilized biological molecules attached to the surface of a transducer that allows an electronic or optical signal to be converted into an appropriate signal. This type of biosensor could be used to measure glucose, sucrose, lactose, *L*-lactate, galactose, *L*-glutamate, *L*-glutamine, choline, ethanol, methanol, hydrogen peroxide, starch, uric acid, etc., by using specific enzymes.[31,127]

Glucose oxidase is normally used to assay glucose concentration. Glucose sensors are the biosensors that have attracted much interest in both research and applications fields. One particularly important medical application of improved biosensors could be in the treatment of diabetic patients for whom proper levels of insulin and glucose must be maintained. For instance, small implantable devices for sampling blood to determine the levels of glucose and regulate the delivery of insulin could be developed using this enzyme.[39] A great variety of immobilization methods (e.g., encapsulation, entrapment) and transducers have been developed to construct the glucose sensors with better performance and practicability since the work of Clark and Lyons in 1962.[40] Several materials have been used, such as polyethylene terephthalate (PET), polyacrylamide, *N*- isopolyacrylamide, sol-gel, poly(2-hydroxyethyl methacrylate), alginate, artificial resins, glass, etc.[31,78,132]

For example, Zhang and Cass[133] have also immobilized alkaline phosphatase on a nanoporous nickel-titanium film for sensor applications.

A number of other enzymes have been described with great potential for medical applications, including carboxypeptidases, collagenase, fibrinolysin, pepsin,[44] streptokinase,[45] subtilisin, thrombin, tissue plasminogen activator, α-amylase, α-galactosidase, glucoamylase, lactase (-galactosidase), pectinase, pancreatin, phospholipases, cholesterol esterase and other DNases, RNases, phosphatases, esterases, sulfatases, isomerases, glucose isomerase, superoxide dismutase, cholesterol esterease, creatine kinase, and penicillin acylase.[45,58,114,134–136]

17.3.2.5 Advantages and Disadvantages of Immobilized Enzymes

The use of immobilized enzymes normally offers several advantages over free enzymes, such as increased stability, localization, and retention of the molecules at the material surface, which enables easier handling, repeated use, and decreased cost. Other important advantages of using therapeutic immobilized enzymes are the prolonged blood circulation lifetime without the loss of specific activity[9] and the lower immunogenicity.[11,137] This advantage is particularly important for delivering enzymes or other biomolecules and may constitute an alternative and suitable method for the enzyme replacement therapy. However, some limitations have been attributed to the use of immobilized enzymes in biomedical applications, such as mass transfer resistances (substrate in and product out), adverse biological responses of enzyme support surfaces (*in vivo* or *ex vivo*), fouling by other biomolecules, greater potential for product inhibition, and sterilization difficulties.[126,137] Although the preparation of sterile immobilized enzyme systems may be complex, sterilization may be achieved by filtrating all the reagents and protein solutions through 0.2-μm filters and working under aseptic conditions.

A very important issue regarding the use of enzymes or other products derived from biological or biotechnological processes in medical applications is to ensure that these therapeutic products do not contain any pyrogenic material, toxins, or infectious agents able to cause harmful effects.[138] For that, it is necessary to perform a complete examination of the products to test their safety in terms of local tolerance, toxicity, carcinogenicity, and immunogenicity, among other pharmacological safety tests.[138,139] Taking into account the diversity in the range of products and the uncertainty about the regulatory status of some of them, it is necessary to design safety evaluation programs to provide useful information to the responsible of clinical trials and to ensure patient safety.

17.4 CONCLUSIONS AND FUTURE PERSPECTIVES

Enzyme instability, combined with the high cost associated with their isolation and purification, had been restricting the general use of therapeutic enzymes on a clinical basis. With the advances made in recombinant DNA technology, it is possible, by means of using adequate expression systems, to put available in larger quantities many enzymes, both for the assay of metabolites and for enzyme replacement therapy. The immobilization of enzymes on support materials had contributed largely to the success of diagnosis and enzyme therapy approaches.

The recent progress in biological science had revealed many types of therapeutic proteins able to regulate various cell functions. On the other hand, the development of new immobilization strategies, such as selective immobilization of proteins to self-assembled monolayers presenting active site-directed capture ligands[140] or protein immobilization within specific locations (protein patterning),[82] may constitute the basis of future immobilization methods. This is particularly important for biomedical applications where it is necessary to control the densities of immobilized proteins, the binding strength, and, most important, their binding orientation. The success of therapeutic agent delivery strategies will be mainly dependent on the developments in these research fields.

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18 Use of Chemically Modified Chitosan and Other Natural-Origin Polymers in Tissue Engineering and Drug Delivery

E. T. Baran and Rui L. Reis

CONTENTS

18.1 INTRODUCTION

Chitin is a copolymer of *N*-acetyl-glucosamine, natural polymer harvested mainly from the exoskeleton of crustaceans such as crabs and shrimps. The deacetylated form of chitin *N*-glucosamine, namely chitosan, can be readily dissolved in dilute acids and is then more accessible for utilization in chemical reactions (Figure 18.1). Chitosan has tree kind of reactive functional groups, at the C-2, C-3, and C-6 positions. Chemical modification of these groups can provide numerous materials with different physical properties for different biomedical applications. By means of inter- and intramolecular hydrogen bonding, chitosan can give origin to excellent films and fibers. Chitin and chitosan have been used as physical barrier to prevent postsurgical adhesions,[1] artificial skin,[2] hemodialysis membranes,[3] wound dressing, and suture materials.[4] These materials can combine a reasonable biocompatibility with an intrinsic mechanical performance.

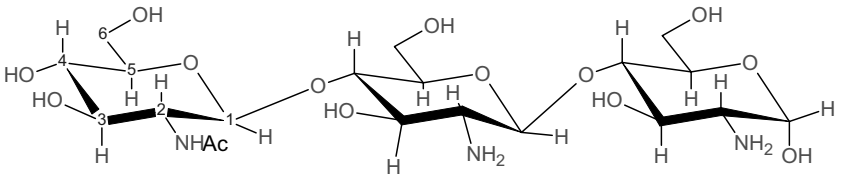

FIGURE 18.1 Chemical structure of chitin and chitosan (degree of acetylation [C-2] considered greater than 50% for chitin).

Certain medical applications of chitin and chitosan require physical stability and integrity at surgical site. Glutaraldehyde has been frequently used to crosslink chitosan and was in fact among the first chemical reagent to be able to form a chitosan gel for the immobilization of enzymes.[5] Recently, attention has been focused on the use of nontoxic crosslinkers of natural origin, for example genicipidin[6] oxidized glucose[7] and oxidized soluble starch.[8] Stabilization of chitosan succinate and hydroxamated chitosan succinate by iron (III) by means of ionic crosslinking can also be used to prepare hydrogel beads for prolonging drug release.[9] Ion complexes of chitosan, such as the ones with nontoxic citrate, malate, and oxalate counter-ions, can provide stable hydrogels with pH-sensitive,[10] sustained drug release,[11,12] and thermosensitive[13] properties. Chemical modification of functional groups of chitosan may change their physical, mechanical, chemical, and biological properties. Especially, the biological properties, which comprise biodegradability, hemocompatibility, and cytocompatibility, are an essential factor for the tissue engineering and drug delivery fields and they can be clearly improved by derivitization of chitin and chitosan with suitable functional groups.

18.2 CONJUGATION WITH IONIC GROUPS

18.2.1 Sulfated Chitosan and Chitin

Because of the structural similarity between heparin molecule and highly sulfated glucosaminoglycan polysaccharides, the sulfate modification of chitosan is attracting great interest being aimed to find an alternative substitute for expensive anticoagulant drugs. Anticoagulant activity of the sulfated polysaccharides results from strong interactions between negatively charged sulfate groups and specific positively charged peptide sequences.[14] Besides negatively charged sulfate groups, introduction of carboxyl groups can further increase anticoagulant activity.[15] For example, antithrombin activity of the polysulfate chitosan synthesized by sulfation of the activated polymer, showed increased antithrombogenic activity, increasing with increasing degree of substitution of the sulfur.[16] The anticoagulant activity of sulfate substituted chitosan (at C-2, C-3, and C-6) prepared by chlorsulfonic acid was observed with the same mechanism of action observed for therapeutic heparin: mainly by antithrombin III-mediated inhibition of FXa.[17]

The introduction of acyl groups, *N*-propanoyl-, *N*-hexanoyl-, and *N,O*-quaternary substitution to chitosan sulfate can further increase anticoagulant activity by means of decreasing positively charged density of the amino groups.[18] The propanoyl and hexanoyl groups increased the activated partial thromboplastin time activity, and the propanoyl groups also increased the thrombin time (TT) anticoagulant activity slightly, while the *N*,*O*-quaternary chitosan sulfate showed only a slight TT coagulant activity.

Sulfonic acid substitution may result in favorable blood-contacting responses including anticoagulant, nonthrombogenic, and reduced complement activity. *N*-Sulfofurfuryl chitosan was obtained by reacting chitosan with the 5-formyl-2-furansulfonic acid.[19] About 23% of the D-glucosamine residues of chitosan could be converted into the sulfofuryl derivative. *N*-sulfofurfuryl chitosan was found to be soluble in aqueous medium over a range of pH from 2 to 12. In addition,

the number of adherent platelets and the extent of platelet activation were significantly reduced on *N*-sulfofurfuryl chitosan as compared with unmodified chitosan.

Chitosan–thioglycolic acid (chitosan-TGA) conjugate material was evaluated to be used as a scaffold material for tissue engineering application by Kast et al.[20] TGA was introduced to chitosan via amide bond formation mediated by carbodiimide. Due to the immobilized thiol groups (240 μmol/gram polymer), the viscosity of the polymer was found to be increased and a transparent gel was formed. The introduction of thiol groups seemed to have no toxic effects on L-929 mouse fibroblast cells.[20] Since chitosan-TGA conjugate hydrated in water is liquid at room temperature and is rapidly gelling at 37°C, it may well be a promising candidate to be used as injectable scaffold material at the site of tissue damage.

Sulfated chitosan derivatives may show different biological activities besides being antithrombogenic. The regioselective syntheses of sulfated analogues of chitin and chitosan are described in relation to studies on structure and biological activity.[21] Fully protected, soluble derivatives of chitosan were found to be useful intermediates for the syntheses of a class of sulfated polysaccharides, 2-acetamido-2-deoxy-3-*O*-sulfo-(1-4)-β-*D*-glucopyranan (3-sulfate, 3S, 4) and (1-4)-2-deoxy-2-sulfoamido-3-*O*-sulfo-(1-4)-β-*D*-glucopyranan (2,3-bisulfate, 23-S, 3). When these compounds were tested for their activities in inhibiting HIV-1 replication *in vitro* and inhibiting blood coagulation, the results revealed that the selective sulfating at O-2 or O-3 showed a much higher inhibitory effect on the infection of AIDS virus *in vitro* than that by the known 6-*O*-sulfated derivative (6-sulfate, 6S). Chitosan sulfoderivatives, *N*-succinylated chitosan sulfate and chitosan-*O*-sulfate, adsorbed on silica substrates were used in removing lipoproteins from plasma as an extracorporeal therapy.[22] The sorption capacity of sulfoderivitized matrix was reported as high as that of sorbents with immobilized heparin.

18.2.2 Incorporation of Phosphate Groups

It was indicated that the chelating ability of dicarboxymethylchitosan (DCMC) interfered effectively with the well-known physicochemical behavior of magnesium and calcium salts.[23] Dicarboxymethyl chitosan formed self-sustaining gels upon mixing with calcium acetate, as a consequence of calcium chelation. DCMC mixed with calcium acetate and with disodium hydrogen phosphate in appropriate ratios (molar ratio Ca/DCMC close to 2.4) yielded a clear solution, from which, after dialysis and freeze-drying, an amorphous material was obtained containing an inorganic component of about one-half of its weight. In a sheep animal model, bone tissue regeneration and complete healing of otherwise nonhealing surgical defects were detected.[23]

N-methylene phosphonic chitosan can be prepared using phosphorus acid at high temperature (70°C) for 6 h.[24] By using ^{1}HNMR spectrum, the introduction of NH-CH_2-PO_3H_2 group replacing the free amino group was demonstrated. Later, the same research group determined degree of substitution ranging 0.72 to 1.57, depending on reaction time (7 to 30 h).[25] The chemically modified chitosan considered in this study film forming ability of its parent chitosan with improvement of an increased solubility over an extended pH range.

Water-soluble phosphorylate chitosans (P-chitosan) (phosphorylated by phosphorus pentoxide in methanesulfonic acid) were incorporated on two calcium phosphate cement formulations, monocalcium phosphate monohydrate with calcium oxide in phosphate buffer and dicalcium phosphate dehydrate with calcium hydroxide.[26] Histological and histomorphological studies from rabbit tibia proved that P-chitosan-containing cements are biocompatible, bioabsorbable, and osteoinductive. A negative relationship between P-chitosan content in cements and their biodegradation rate (lower and medium contents absorbed in 16 weeks) was also reported by the same research group.

Electrostatic interactions between multivalent ionic phosphate crosslinkers, pyrophosphate (Pyro) and tripolyphosphate (TPP) and chitosan may modulate film swelling and in turn drug release. Compared with TPP/chitosan films, Pyro/chitosan films exhibited much better pH-sensitive

swelling and controlled release properties, as determined by model drug riboflavin, due to their relative weak electrostatic interaction.[27]

18.2.3 Other Ionic Groups

EDTA (ethylenediaminetetra acetic acid) conjugate with almost quantitative modification of all amino groups can be prepared between carboxyl of EDTA and amino groups of chitosan in the presence of carbodiimide.[28] The conjugate was found to bind zinc and calcium, which are essential for hydrolytic activity of some proteolytic enzymes. Zinc proteases aminopeptidase N and carboxypeptidase A were observed to be inhibited strongly.[28] EDTA conjugate of chitosan has potential use in protecting perorally administrated therapeutic peptides because of increased proteolytic resistance.

Amino group of *D*-acetyl-glucosamine subunits of chitosan molecule can be reacted with 3-chloro-2-hydroxypropyltrimethylammonium chloride to give rise to quaternary ammonium derivatives to increase ionic interaction ability of the biopolymer. The quaternized macro porous chitosan beads displayed high adsorption of indomethacin drug relative to native beads due to a strong interaction of $-COO^-$ of drug molecule with $-N^+(CH_3)_3$ group of the quateraminated chitosan.[29]

By means of reacting chitosan with silicon ethoxide, membranes with high oxygen permeability and increased cell adhesion properties can be obtained. Silica-chitosan membranes with 50% silica content was reported to be the best material for artificial skin applications in terms of supporting cell growth of fibroblast-like cells (L-929), membrane's oxygen permeability, and conserving tensile strength and flexibility of the membranes.[30]

18.3 ACYLATED AND ACETYLATED DERIVATIVES

Conjugation of chitosan with trimethylchloride can increase permeation and absorption of neutral or cationic drugs as well as hydrophilic macromolecules from intestinal epithelia.[31] Trimethylchitosan (TMC) with a degree of quaternization degree between 22 to 49% showed increased permeation of [^{14}C]mannitol, directly proportional with increased modification, across rat intestinal membrane.[32] This increase in permeability is believed to be the result of reversible opening of tight junction of epithelial cells by charged quaternized chitosan molecules, which is not possible for native molecule.[33,34] TMC60 (60% substitution) has been proven to be a potent enhancer for both nasal and rectal insulin absorption in rats at neutral pH values where chitosan is not effective.[35]

The use of biocompatible macromolecules together with chitosan may provide scaffold material with multifunctional biological properties. Blends of the naturally occurring polysaccharides, cellulose, and chitosan, obtained in the solid phase by the combined action of high pressure and shear deformation, can provide blending of polysaccharides at the molecular level. A mechanism of cellulose–chitosan blend formation in the presence of a diepoxide, used as a crosslinking agent, was studied by Rogavina et al.[36] It was established that the crosslinking agent reacts predominantly at the amino groups of chitosan, with the formation of a three-dimensional network, cellulose macromolecules being located within and partially bound with this network by the crosslinks. Chitosan–gelatin scaffolds were modified with incorporation of hyaluronic acid through crosslinking with carbodiimide and *N*-hydroxysuccinimide.[37] Incorporation of hyaluronic acid improved flexibility of biomaterial and promoted adhesion of human fibroblasts over the biomaterial.

The introduction of succinyl group into chitosan at the N-position of the glucosamine unit is found to be useful for production of water-soluble and less biodegradable materials in the body.[38] As a potential water-soluble drug carrier, succinyl-chitosan (Suc-chitosan) was investigated for its systemic retention and tumor accumulation.[39] Suc-chitosan showed a high plasma half-life (100.3 h) in normal mice, and this was found less (43 h) in tumor-bearing mice (Sarcoma 180), indicating preferential tumor accumulation of the macromolecule.

Acrylic grafts of chitosan are possible means of creating hydrophilic and mucoadhesive polymers, as it has been reported recently.[40–43] Template polymerization of acrylic acid (pAA) in chitosan solution can lead to inter- and intramolecular linkages between carboxyl group of poly(acrylic acid) and positively charged amino groups of chitosan.[40,42] Nanoparticles of chitosan-(pAA) grafted polymer have been proposed as a hydrophilic drug carrier for hydrophilic drugs and sensitive proteins.[40]

There are interesting chitosan derivatives that can be generated by acetylating and acylating chitin and chitosan for biomedical applications.[44] Derivatives of partially *N*-deacetylated chitin (DAC) were prepared via ring-opening reactions with various cyclic acid anhydrides in lithium chloride/*N*, *N*-dimethylacetamide (LiCl/DMAc) system.[45] From the enzymatic studies, the glycosyl bond of succinyl and maleoyl DAC-20 (20% DAC) was found to be degraded rapidly by lysozyme or chitinase, though that of phthaloyl DAC-20 was not. Similar increased susceptibility of acetylated chitosan to lysozyme was shown before,[46] suggesting an increased susceptibility of acetylated derivatives of chitosan to enzyme degradation. The ester linkage of succinyl DAC-20 was found stable against lipase for five days at room temperature.[45]

Selective *N*-acetylation of chitosan with various anhydrides, acetic, propionoic, *n*-butyric, *n*-valeric, and *n*-hexanoic anhydrides with degree of *N*-acylation of about 20–50% was reported.[47] These *N*-acylated chitosan derivatives showed more blood compatibility by blood clotting test, especially *N*-hexanoyl chitosan, and their susceptibility to lysozyme was found to be as high as *N*-acetyl chitosan. The degree of acetylation can play important roles in cutaneous cell adhesion and proliferation. The higher the degree of acetylation of chitosan, the lower the cell (fibroblast and keratinocytes) adhesion on the films.[48] Furthermore, the proliferation of keratinocytes has also been found to be decreased with the degree of acetylation. The next step for these synthetically acetylated and acylated chitosan derivatives would be the evaluation of attachment and proliferation of cells on the biomaterial's surface.

It is desirable to inhibit growth of the keloid fibroblast and to promote growth of the normal skin fibroblast during wound healing.[49] A water-soluble carboxymethyl derivative of chitin carboxymethyl-chitosan (CM-chitosan) showed two-fold bioactivities in the growth of normal skin fibroblast and inhibition of keloid fibroblasts.[50]

Acid chloride and acid anhydride surface modification of chitosan films may change hydrophobicity, depending on the polarity of the substituted group. The surface became more hydrophobic than that of nonmodified films when a stearoyl group ($C_{17}H_{35}CO$-) was conjugated to the surface while the surface found to be more hydrophilic with succinic anhydride or phthalic anhydride treatment.[51] Hydrophobic films also promoted bovine serum albumin and lysozyme adsorption.

Regioselective carboxylation of chitosan at C-6 is possible by oxidation of NaOCl, in the presence of Tempo® and NaBr.[52] The resulting anionic polymer was found to be soluble over the entire pH range and having metal chelating property. The oxychitin–chitosan complex was found to be degraded by lysozyme, lipase, and papain while other enzymes were found to be ineffective.[53] When this complex was tested as bone prosthesis coating in an animal model, it was found to be biodegradable and it lead to bone formation at the bone-prosthesis interface. C-6-carboxylated chitosan by selective perchloric acid oxidation in the presence of chromium trioxide can be used for further selective modifications to obtain unique derivatives. Such as, after Schiff base reaction of carboxylated polymer by *p*-chlorobenzaldehyde in methyl alcohol, selective *O*-acetylation with acetic anhydride on C-3 of the carboxylated chitosan is possible to produce analogs of bacteria antigen polysaccharides, which are acetylated at C-3.[54]

Amphipathic polymers such as poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO) have also been successfully used with natural and synthetic macromolecules to obtain hydrophilic biocompatible biomaterials. The interactions that lead to surface-induced thrombosis, surface modification with water-soluble polymers, such as PEG or PEO, can prevent plasma protein adsorption, platelet adhesion, and thrombus formation by the steric repulsion mechanism.[55] To improve blood compatibility, chitosan surface was modified by the complexation-interpenetration method using an anionic derivative of poly(ethylene glycol). On PEG-modified chitosan, much less contact-

adherent platelets could be detected compared with unmodified polymer, showing increased blood compatibility of modified polymer.[56]

PEG dialdehyde of different molecular sizes were crosslinked with partially reacetylated chitosan via Schiff reaction and hydrogenation of aldimines generated water-soluble polymers, which can be aggregated to yield insoluble soft spongy biomaterials.[57] When exposed to papain and lipase, a progressive dissolution of the biomaterial was reported, while no dissolution with lysozyme, collegenase, and amylase was observed. The material was found to be biocompatible and not toxic over human colon carcinoma enterocyte-like cell line (Caco-2).

Graft copolymerization of PEG diacrylate on chitosan backbone was achieved by Shantha et al.[58] using ceric ammonium nitrate initiation technique. Microspheres were prepared from graft polymer by polymer dispersion technique. These microspheres appeared to be hydrophilic and form aggregates during their preparation. Tetraethylene glycol was modified by two different approaches to synthesize the scaffold of dendrimer. Poly(amido amine) (PAMAM) dendrimers (G = 1–3) having tetraethylene glycol spacer were prepared and attached to chitosan by reductive *N*-alkylation. On chitosan molecules, the degree of substitution of dendrimers was 0.03–0.18. Sialic acid residue bound PAMAM dendrimers of each generation were successfully attached to chitosan.[59] The mechanical properties were slightly improved with the proper amount of PEG, but the improvement was not obvious and was destroyed by the wrong proportion of PEG. Cell culture studies and amounts and structures of the adsorbed proteins on different materials showed that the PEG effectively improved the biocompatibility of the materials. The PEG enhanced the protein adsorption, cell adhesion, growth, and proliferation, but the effects were impaired by excessive PEG amounts.[60]

18.4 CONJUGATION WITH BIOLOGICAL MOIETIES

18.4.1 Monosaccharide Derivatives

Organosoluble and branched chitin and chitosan derivatives have been prepared with various sugars, *D*-mannose, *D*-galactoside, *D*-maltodiose, and *N*-acetyl-*D*-glucosamine.[61] Conjugates were found to be susceptible to lysozyme degradation. All the branched products were found soluble in all common organic solvents. Due to the branched nature of products, some unique bioactivities can be expected from these conjugates like antitumor, immunoadjuvant activity, hypolipidemic activity, antimicrobial activity, and biodegradability.

The structural similarity of chitosan to glycosaminoglycans (GAGs), which are components of extracellular matrix, makes this material suitable for hepatocyte culture. Further interaction with hepatocytes can be induced by conjugation of some sugars molecules specific to hepatocyte cell membrane receptors. For instance, fructose, which is specific to asialoglycoprotein receptor in hepatocytes, was reacted between aldehyde in sugar and amino group in chitosan with 34% conjugation efficiency.[62] Within the fructose-modified chitosan scaffold, cell organization and increased cellular interaction were detected as compared to the unmodified scaffolds. Metabolic activities in terms of albumin secretion and urea synthesis were much higher on the fructose-modified chitosan. Galactosylated-chitosan-dextran-DNA was used by Park et al.[63] for liver targeted-delivery system studied. This system was found to be efficient to transfect liver cells expressing asialoglycoprotein receptor, which specifically recognized the galactose ligand on chitosan. In a similar study, lactobionic acid-bearing galactose group was coupled with chitosan for liver specificity and PEG for long circulation time.[64] The DNA-modified-chitosan complex has only transfected Hep G2 (human hepatocellular carcinoma cells) having asialoglycoprotein receptors.

Lactose conjugated *N*-succinyl-chitosan biodistribution was studied after intravenous administration to mice inoculated with M5076 tumor cells.[65] Conjugate showed long circulation and localized specifically to liver, proposed as potential drug carrier at early stage of metastasis. Lactose and azide (*p*-azidebenzoic acid) moieties have been coupled to chitosan by condensation reaction in order to obtain water-soluble and photocrosslinkable hydrogel.[66] Lactose moiety in this conjugate

was used to increase water solubility. Upon activating photoreactive azide residue by UV radiation, wound closure and acceleration of healing have been detected on animal models. Binding strength obtained with an aqueous solution of functionalized chitosan over skin cuttings of mouse tail was comparable to fibrin glue and the curing time was detected much shorter than fibrin glue. The reactive chitosan hydrogel, due to its ability to accelerate healing, has potential as a dressing for wound occlusion and tissue adhesive in urgent hemostasis situations.

18.4.2 Conjugation with Lipid and Other Biomolecules

The conjugation of lipid groups to chitosan molecules can create an amphoteric self-assembling molecule, which can be used as drug delivery carriers. In one such study,[67] chitosan derivative surfactant, *N*-lauryl-*N*-methylene phosphonic chitosan, has been synthesized by conjugating alkyl group with free amino group of polymer via reductive amine chemistry with a degree of substitution of 0.33. The conjugate was reported to be dissolved in organic solvents at high temperature and have the ability to form emulsion in aqueous solution. This possible potential may be used for solubilization of hydrophobic drugs in drug delivery applications.

Palmitoyl glycol chitosan was synthesized by reacting palmitic acid *N*-hydroxysuccinimide in ethanol solution.[68] This conjugated polymer can be converted into quaternized form by dispersing palmitoyl glycol in pyrrolidone overnight and further reaction with methyl iodide and sodium iodide in alkali solution. The amphiphilic polymer was shown to be nonhemolytic when present as the liquid solution and relatively noncytotoxic, presenting a potential for solubilizing and entrapping of hydrophobic drugs by means of micelle-forming ability.

Palmitoyl glycol chitosan (GCP) hydrogel has been evaluated as an erodible controlled-release system for the delivery of hydrophilic macromolecules.[69] As the degree of amphiphilic palmitic acid increased the hydrophobicity, and as a result slower release of model drug was reported, probably due to decreased water entrance and unfavorable diffusion of drug from the gel. In their recent study, the same research group investigated the buccal absorption of denbufylline from this mucoadhesive GCP hydrogel, using the rabbit animal model and Carbopol (CP), denbufylline, used as control mucoadhesive polymers.[70] Sustained release of drug was observed for at least 5 h after dosing from GCP hydrogels, as compared to 1 h release from control, showing retarding effect of palmitoylation.

Deoxycholic acid, which is the main component of bile acids, was used to modify chitosan hydrophobically and to obtain self-assembling macromolecules.[71] The transfection efficiency of self-aggregate DNA complex from deoxycholic acid-modified chitosan was shown to enhance the transfection efficiency over monkey kidney cells. Chitosan containing 5.1 deoxycholic acid groups per 100 anhydroglucose units was synthesized by a carbodiimide (EDC)-mediated coupling reaction. The feasibility of chitosan self-aggregates for the transfection of genetic material in mammalian cells was also investigated.[71] Self-aggregates have a small size (mean diameter of ca. 160 nm) with a unimodal size distribution. Self-aggregates can form charge complexes when mixed with plasmid DNA. This physical property of deoxycholic-chitosan can be used as self-aggregating nonviral gene delivery system.

The adhesion and growth of epithelial cells (ECs) can be supported by grafting surfaces with Gly-Arg-Gly-Asp (GRGD) cell adhesive peptide.[72] Grafting of GRGD-SANPAH (*N*-succinimidyl-6-[4-azido-2-nitrophenylamino]-hexanoate) to chitosan surfaces by surface adsorption and subsequent ultraviolet irradiation for photoreaction was studied by Chung et al.[73] The adhesion and growth of ECs was poor on native chitosan surface and the surfaces grafted with GRGD have been shown promoting growth rate about 50% compared with native polymer.

The pancreatic proteolytic enzymes such as trypsin, chymotrypsin, and elastase are responsible for the degradation of perorally given peptide or protein drugs before reaching their target. By means of covalent attachment of trypsin inhibitors to drug carriers, adsorbed and encapsulated protein drugs can be protected from proteolytic attack of luminal enzymes during their transport

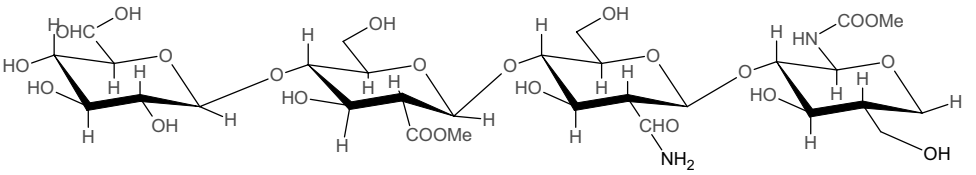

FIGURE 18.2 Chemical structure of hyaluronic acid.

in gastrointestinal tract. In one example of this kind of strategy, antipain was conjugated to amino group of chitosan using carbodiimide activation method and a significant inhibitory effect was observed against trypsin degradation.[74] A controlled insulin release was detected from antipain-chitosan without loosing polymer's mucoadhesive property.

18.5 MODIFICATION OF AMINOGLYCAN POLYSACCHARIDES

Due to the structural similarity of glycosylaminoglycans with chitosan molecule, it would be useful to mention recent applications of chemically modified hyaluronic acid (HA) and its sulfated counterpart, chondrotin sulfate. These hydrophilic biopolymers have been already shown suitable for biomedical applications, such as for drug delivery and tissue engineering scaffolds.[75]

Hyaluronan, formerly known as hyaluronic acid, is a main glycosaminoglycan distributed in the extracellular space. It is linear polymer of glucoronic acid and *N*-acetylglucosamine (Figure 18.2). The hydrophilic, polyanionic surfaces of hyaluronic acid materials present not favorable surfaces for the attachment of cells, which adhere better to polycationic and hydrophobic surfaces. To improve the stability of the polymer, esterification of the free carboxyl group of glucuronic acid with different type of alcohols was studied.[76] By masking the free carboxylic group, the anionic charge of hyaluronan can be decreased and the hydrophobic parts along the chain may be increased. The highly benzyl ester derivative of hyaluronan (HYAFF®) above threshold of 80% of esterification material was noted to be stable and less hydrated, and in turn allowed fibroblastoid cell adhesion and spreading.[77]

To improve the cell attachment and convert cell-resistant surface to one that stimulates cell adhesion, spreading, and proliferation, hyaluronic acid-based biomaterials coupling with biopolymers containing RGD sequences have been proposed. In one such study, thiolated collagen and hyaluronic acid component were synthesized using hydrazide-carbodiimide technology and blended composite crosslinked in air to give disulfide crosslinked hydrogel film.[78] Balb/c 3T3 fibroblast cells were reported to be attached and spread on the hydrogel surface.

Controlled modification of the carboxylic acid moieties of hyaluronic acid with mono- and polyfunctional hydrazine have also been tried in order to obtain slow-release drug delivery systems and use as tissue engineering scaffold.[79] In the presence of carbodiimide, mono-, bis-, and poly-hydrazides can be attached to hyaluronic acid, and these reactive groups can be used further for the attachment of therapeutic drugs or bioactive moieties.

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Part IV

Biocompatibility and Immunological Responses to Degradable Biomaterials

19 Cytotoxicity Screening of Biodegradable Polymeric Systems

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CONTENTS

19.1 AIMS OF CYTOTOXICITY SCREENING IN BIODEGRADABLE POLYMERIC SYSTEMS

The technological advance in the materials production methodologies and the large progress in the development of prosthetic devices with improved implantation performance were made efficient by the parallel establishment of adequate strategies for testing the biofunctionality and the biocompatibility of the devices. The evaluation of the biological response to the materials should follow procedures that allow for the objective evaluation of the devices safety and biocompatibility.[1,2]

The most important requirement for a biodegradable polymer to be used in medical applications is its compatibility not only in terms of physical and chemical properties, but also in those that define their behavior at the time they contact the body.

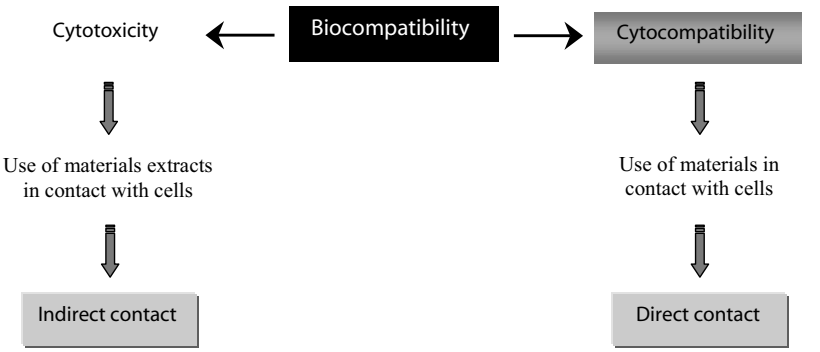

FIGURE 19.1 Relationship between biocompatibility, cytotoxicity, and cytocompatibility. The cytotoxicity testing relies more on biochemical tests, while morphological evaluation is frequently used when cytocompatibility is being evaluated.

In terms of biocompatibility, the requirements that degradable materials must fulfill are much more demanding than those needed from nondegradable materials.[3] In fact, in addition to the potential problem of toxic contaminants leaching out from the implant, such as residual monomers, stabilizers, emulsifiers, and many other types of additives, it is also necessary to consider the potential toxicity of the degradation products and subsequent metabolites.[3] Every material that aims to be used in biomedical applications needs to be screened for its biocompatibility. The biocompatibility of a material, while defined as the answer of cells to contact with a material or with its leachables, can be equated with the characteristics of degradation and toxicity.[4] In other words, a biocompatible material should not influence negatively the organism nor be influenced by the surrounding environment while performing a particular function.[1,5,6] Figure 19.1 defines the relations between biocompatibility, cytotoxicity, and cytocompatibility.

Cytotoxicity tests are important for the screening and evaluation of biocompatibility of "substituting organs"[7] such as implantable biomaterials, and cytotoxicity testing represents the initial phase in testing biocompatibility of potential biomaterials and medical devices. Its purpose is to act as a reliable, convenient, and reproducible screening method to detect, at an early stage in the testing process, cell death or other serious negative effects on cellular functions.[8–16]

When being evaluated for safety, biomaterials should be considered according to their predicted use or particular prosthetic device. In this sense, biocompatibility means not only absence of a cytotoxic effect but also positive effects in the sense of biofunctionality,[1,17–19] i.e., promotion of biological processes which further the intended aim of the application of a biomaterial.

Therefore, in order to assess the biocompatibility of a particular material, it is typical to undertake the consecutive execution of different tests.[8,9,11,15,16] The results, collected together, aid in defining the biological tolerance to the materials.

In vitro cell culture studies are a very sensitive method for testing the toxicity of soluble polymers and their degradation products. These tests have the advantage of being relatively well controlled and are generally accepted[1,8] as a very effective method for biocompatibility and toxicity testing.

19.2 BIODEGRADABLE POLYMERIC SYSTEMS — EFFECTS OF DEGRADATION AND LEACHABLES IN BIOMEDICAL APPLICATIONS

Cytotoxicity deals mainly with the substances that leach out of biomaterials.[11,20,21] For example, polymers often have low-molecular-weight "leachables" (additives, low-molecular-weight compo-

nents, initiator fragments, others) that exhibit varying levels of cytological and physiologic activity responsible for cell toxicity, alteration of metabolic balances, or more severe difficulties.[10,11,21,22]

In the case of biodegradable systems, the action of body fluids containing enzymes that cause the degradation of the material is an important feature that should be well studied. Degradation profiles of the proposed biomaterials should be established in order to determine the behavior of the materials when in contact with the body. Many cytotoxicity tests use the so-called extracts from the materials, which are constituted by cell culture medium and the possible leachables of the material when in contact with the cell culture medium. The extract content is primarily determined by the duration of the extraction procedure, as well as by the morphology of the material. In our group, the study of starch-based materials reveals that compact samples, porous scaffolds, and microparticles present a quite distinct leaching profile, which depends of course on the processing methodology. For instance, the degradation profile of tissue engineering starch-based materials processed as scaffolds can be found in a work by Reis et al.[23] As for starch-based microparticles, the degradation profile yielded a much-increased amount of reducing sugars (an indication of starch degradation) during the 90-day evaluation.[24]

It is clear that the morphology of the system to be tested greatly influences the performance of the materials in the cytotoxicity screening tests. The larger the surface area, the greater the degradation rate, so it is obvious that greater degradation will yield a greater amount of leachables from the system, that can of course have an effect on the cytotoxicity of the materials.

19.3 TESTING POLYMERIC BIODEGRADABLE SYSTEMS

19.3.1 *IN VITRO* VERSUS *IN VIVO* TESTING

The major advantage of using *in vitro* methods for cytotoxicity testing is the comparative cost-effectiveness and speed of the tests, which make them particularly suitable as a tool for screening large numbers of potential biomaterials and their modifications,[8,10,11,19] allowing for a standardization/reproducibility of experimental conditions. This becomes even more relevant due to the background of current public (and expert) opinion that leads to a pressure for the reduction of animal experimentation whenever that is possible.[8,10,11,19,22,25] Coupled with this is the high sensitivity of the methods, which enables researchers to identify potentially cytotoxic materials, at an early stage in the testing procedure.[15,26,27] In general, *in vitro* cytotoxicity tests exceed the sensitivity of *in vivo* tests of acute systemic toxicity and intracutaneous irritation. This is because the *in vitro* assays are target cell assays, i.e., they make use of the most sensitive cells. In contrast with *in vivo* tests, the *in vitro* tests minimize the variables of metabolism, distribution, and absorption, allowing the maximization of dosage per cell of any toxic substance present to produce a highly sensitive test system.[25] Regarding all these practical aspects, the degree of *in vitro* toxicity of a biomaterial is almost proportional to its *in vivo* compatibility.[1]

At the top of the list of disadvantages of *in vitro* methods is the fundamental problem of extrapolation to the *in vivo* situation and, in particular, to humans.[1,15,19,25,28] This problem cannot be solved by any amount of philosophical discussion, and thus it should be stressed that *in vitro* testing represents always only one phase in studying biocompatibility.[1,15,19,25,29] The specimens classified as *in vitro* biocompatible must enter a further phase of testing, which requires *in vivo* observation and obtaining direct data from complex tissue systems.[15] A second limitation not to be underestimated is the problem of *in vitro* specific sensitivity, which simply describes the phenomenon of deleterious effects *in vitro*,[15] which do not occur in the intact organism. In other words, *in vitro* test systems may lead to false negatives. However, this disadvantage should not be regarded as serious, as it is unlikely that a material that causes rapid cell death in all cell types tested, both cell lines and primary isolated cells, will prove to be totally harmless *in vivo*. Third, *in vitro* methods may be restricted by the choice of cell type.[15] This problem must be considered as more serious if only one cell line is used in the screening methods. Therefore, the use of primary cultured epithelial

and mesenchymal cells should be favored, in addition to the use of a few established transformed cells in the form of cell lines.

Furthermore, stagnant *in vitro* culture systems should be avoided in order to overcome the accumulation of metabolites and toxic products.

A further disadvantage is the failure of *in vitro* methods in providing adequate information on biomaterial degradation.[15] This type of information can only be collected by studying host–biomaterials interactions *in vivo*. Nevertheless, it is possible to study limited, yet important, aspects of this matter by constructing simulation experiments *in vitro.*

Complete safety/toxicity information should result in series of tests preferentially involving initial *in vitro* screening and finalized by *in vivo* methodologies. In both cases, experimental information should cover two different aspects: the materials response and the host behavior.[30]

19.3.2 Cell Lines versus Primary Cultures in *In Vitro* Testing

The need to restrict animal experimentation to a minimum enhanced the necessity to use *in vitro* systems to select, adequately, potentially useful biomaterials and those unsuitable for human application. For cytotoxicity analysis, cultured mammalian cells are preferred, since chemicals responsible for the development of diseases and death in animal and human are ultimately excreted at the cellular level.[30]

Using cell lines to perform cytotoxicity screening has become a routine in almost all laboratories around the world. However, it must be stressed that the ideal situation is the use of human, nontransformed cells, that is, of primary isolated cells in early passage.[31–34] Both approaches have advantages and disadvantages; thus, many authors defend the use of cell lines at the first stage of screening and the use of adequate primary cultures relevant to the purpose for which the potential biomaterial has been developed.[31–36]

Primary cell cultures are achieved through enzymatic or mechanical disaggregation of a piece of tissue or by spontaneous migration from an explant and may be propagated as an adherent monolayer or as a cell suspension.[37,38] These cells are generally heterogeneous, with a low fraction of growing cells, but with a variety of cell types representative of the tissue.[9,37,38] The major advantage is their ability to proliferate and replicate although specialized cells and functions can be lost with the propagation of the culture.[9,37,38]

Cell lines are previously established, and generally have origin in transformed primary cultures.[37] While these have limited life, a continuous cell line is immortal and may be derived from different species and tissues.[37] Comparatively with primary cultures of the same type of cells, a cell line presents morphological alterations such as decrease cell size, reduced adherence, and higher nucleus.[9,32,39] The tumor nature of these cells theoretically involves the possibility of phenotype and genotype variations.[9] The main drawback using cell lines can be the extrapolation of results obtained *in vitro* to *in vivo* situations where normal cells act.[31,35,36]

Cell lines have, however, the advantages of being highly sensitive and homogeneous, allowing for its use for a long time period representing an abundant source of cell material.[35,37]

Both cell types can be frozen and retrieved intact, even after many years.

19.3.3 Standardization of Cytotoxicity Screening Tests

The use of standard practices of biological testing provides a reasonable level of confidence concerning the response of a living organism to a given material or device, as well as guidance in selecting the proper procedures to be carried out for the screening of new or modified materials.[40]

The methodologies for cytotoxicity assays are described in several standards published by different entities, such as the American Society for Testing and Materials (ASTM),[33] the British Standards Institute (BSI),[32] and the International Standards Organization (ISO).[34,41]

Cytotoxicity assays differ mainly in the manner in which the test material is exposed to the cells. The choice of the method varies with the characteristics of the test material, the rationale for doing the test, and the application of the data for evaluating biocompatibility.[9,11,25,42,43] Direct contact techniques involve assays in which the material to be tested is brought into direct contact with the cells, usually by seeding a cell suspension onto the material.[11,42,43] Indirect methods are mainly of two types.[11,42,43] The first consists in the separation of the material from the cells by use of a diffusion barrier, such as an agar layer, placed between the material and a cell monolayer.[11,42] The second consists in the addition of an extract of the material to a cell monolayer, which is nowadays probably the most widely used method.[11,42] It is convenient to prepare the extract of the material in the culture medium used for the chosen cell type. Indirect testing techniques are important for the detection of leachable substances, which could exert toxic effects on cells.[11,42]

19.4 SHORT-TERM TESTS VERSUS LONG-TERM TESTS

The established standards for the evaluation of the biocompatibility of potential biomaterials were initially created to evaluate nonbiodegradable materials proposed for biomedical applications.[32,33,41] Thus, several variables emerged in the evaluation of the biocompatibility of those materials. In focus were, for example, the possible effects of the metabolites resulting from the degradation, the local and remote interactions of cells with those products, and the rate and mechanism of degradation.[16,36,44–47] Considering that the results obtained with the standard tests could be influenced by those new parameters, adaptations have been made along the way. Long-term tests were initially considered only *in vivo,* but the study of degradable materials has to predict *in vitro*, as much as possible, the continuous effect of those systems and mainly of their degradation products.[16,36,44–47] Of course it is not possible to maintain *in vitro* cell cultures for indefinite time; thus, the need to mimic long-term degradation launched biocompatibility tests using extracts of the materials obtained under different conditions.[16,41,44,46] High temperature degradation assays are based on the assumption that the degradation of biodegradable materials can be accelerated at high temperatures, thereby releasing products that are expected to be released *in vivo* after long-term implantation.[41,48] However, there is still some controversy about the veracity of these high temperatures having the same influence on the degradation behavior to what might occur *in vivo*. The so-called "real-time degradation tests" were suggested by the ISO standard.[41,44,46] Extracts are obtained at 37°C, body temperature, at different times, which can go up to 52 weeks. Furthermore, since the human body is a dynamic system with constant changes of fluids, simulating the degradation of the materials under movement/shaking was also considered as a way of better simulating the *in vivo* conditions.[41]

19.5 WHAT TO EVALUATE AND TECHNIQUES AVAILABLE

It is generally accepted that the cytotoxicity effect of the materials in investigation can manifest itself in different forms such as cell death, loss of membrane integrity, reduced cell adhesion, altered cell morphology, reduced cell proliferation, and reduced biosynthetic activity.[9,16,49] The numerous techniques currently used can be grouped according to the parameters evaluated — morphological, biochemical, and genetic — even though a parameter prevails: the assessment of cell death, which has been the most widely adopted end-point in measuring the effects of chemical toxicants.[50]

19.5.1 MORPHOLOGICAL

Morphologic changes of cells that were contacting biomaterials or biomaterial extracts indicate their toxicity.[7] However, information on cytotoxic effects is still obtained by subjective visual inspection of microscopic samples.[7] A qualitative evaluation of cell morphology is often based on the examination, by inverted microscopy. Typical characteristics of cytotoxic cellular alterations

include shrinking of the cell nucleus, fragmentation of the cytoplasm, granulation formation, rounding off, and cell detachment.[16,38,39]

Dye exclusion tests make use of certain dyes, such as trypan blue, which can be excluded from viable cells. Thus, in a cell population, cells that stain with the dye are assessed as being nonviable.[11,37,42] This method is, however, not always reliable.[11,36,42] Under certain circumstances, the dye can bind to serum proteins and thus fail to be taken up by injured or dead cells.

On the other side, vital staining tests are based on the ability of viable cells to take up and retain a dye. An example is neutral red,[42] which is endocytosed by viable cells. Damage of cell membrane allows neutral red to be internalized inside lysosomes, allowing for conclusions about cell integrity. The stain can be measured spectrophotometrically and compared with a standard curve for cell quantification.[42]

A combination of the two principles was suggested by Dankberg et al.,[51] using a combination of fluorescein diacetate (FDA) and ethidium bromide (EB). FDA is taken up by intact cells and converted by esterases to the polar compound fluorescein, which remains in the cytoplasm, giving green fluorescence on UV excitation. EB can only penetrate cells with damaged plasma membranes and binds to nucleic acids, resulting in an orange-red fluorescence. Others have used acridine orange (AO) instead of FDA.[42]

In addition to the evaluation of cell morphology and viability, the use of these stainings can also allow for forming an opinion about the cells adhesion behavior. The analysis of the results has, however, to be cautious. Reduction of cell adhesion may be wrongly interpreted since it may not be a toxic effect and has to be distinguished from cell death. If certain surfaces aim to be adhesive for cells working as a substrate for cell proliferation, others will be biocompatible if cells fail to adhere to their surface or to the tissue culture polystyrene plates in the presence of their extracts.[9,16,42] (For more details on this, please see Section 19.6.)

19.5.2 Biochemical

The cell membrane integrity tests are among the most useful methods of quantitative assessment of cytotoxicity and make use of the fact that an intact cell membrane has a selective barrier function, and, in some tests, that certain substances are metabolized by functionally intact cells.[11,42] These are often considered as methods to quantify cell proliferation. A growth curve can be obtained by counting the number of cells in cultivation at different intervals after seeding, which allows extrapolating a cell number from the cytotoxicity test.[11,42] This usually involved enzymatic treatment to obtain a cell suspension, which is then counted.

Alternative methodologies include the quantification of total protein or DNA content of culture, which is linearly correlated with the number of cells.[11,31,42,52–54]

Examples are certain tetrazolium salts, such as MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), which is converted by mitochondrial succinate dehydrogenase to a blue formazan.[11,42,55] This reaction can be quantitated using a spectrophotometer and has been modified to be as sensitive as the uptake of radioactive nucleotides to assess cell proliferation.[11,42] These molecules are taken up into DNA of cells during mitosis, allowing for the identification of new cells by autoradiography. The synthesis of DNA can then be followed, through the incorporation of labeled bromodeoxyuridine (BrdU), a pyridine analogue, and ^{3}H-thymidine.[2,31,36,56] In the case of BrdU, monoclonal antibodies coupled with a visualization system such as peroxidase-antiperoxidase were also used, avoiding then the radioactive precursors.[57]

Lactate dehydrogenase (LDH) is a cytosolic enzyme present within all mammalian cells.[47,58,59] The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid. *In vitro* release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability.[47,58,59] As a result, the release of lactate dehydrogenase has proved to be a reliable test for cytotoxicity testing.

19.5.3 Genetic

About two decades ago, *in vitro* mutagenicity tests were adopted as the first *in vitro* methods in regulatory toxicology.[60] For reasons of animal welfare and better science, many validation studies of various *in vitro* methods were performed to replace the Draize eye irritation test.[60] In order to license a pharmaceutical or chemical, a compound has to be tested for several genotoxicity endpoints, including the induction of chromosomal aberrations *in vitro*.[61] Genotoxicity tests form an important part of cancer research and risk assessment of potential carcinogens,[62] through the assessment of whether a chemical has the potential to cause somatic or germ-cell effects in animals (i.e., the potential to induce cancer or heritable mutation).[63] Such genotoxicity testing is usually undertaken in a stepwise approach: first an assessment *in vitro* to determine intrinsic genotoxic activity, and secondly, an evaluation *in vivo* to determine whether any such activity is expressed in the whole animal.[63] There is a distinction between *in vitro* assays to detect intrinsic genotoxic properties and *in vivo* assays as a subsequent phase to show the realization of this potential in an intact organism.[64]

Genotoxicity tests have had widespread applications in testing pure chemicals,[26] but now they are developing a great interest for applications within biomaterials testing, since the question of whether biomaterials have adverse effects on the body is of major concern,[65] and specific mutation events have been shown to be related to the progression to carcinogenesis.[26,66] As a matter of fact, ISO 10993 lays down specific requirements for biocompatibility, including the tests based on the nature of the contact and the duration of implantation of the biomaterial.[67] The standard stipulates that all materials that will be in contact with mucous, bone, or dentinal tissue if the contact exceeds 30 days, as well as all implantable devices if the contact exceeds 24 h, must undergo genotoxicity testing.[67] Generally, the main focus is directed to immediate influence like cytotoxicity, but especially in the field of dental materials attention was drawn to long-term effects such as genotoxicity and mutagenicity.[65]

Regarding the assessment of genotoxicity of materials to be used in biomedical applications, several tests could be performed, but we will herein only focus on the ones that seem more advantageous for our study case. From a whole range of tests, only the comet assay (or single-cell electrophoresis assay) and the micronucleus assay will be discussed within this chapter.

A basic principle underlies the genotoxicity tests: the agent to be evaluated is placed in contact with the cells during a predetermined time, after which several gene-related features are evaluated, against a positive (using known genotoxic agents) and a negative control. It is readily comparable to the procedure for cytotoxicity tests. In this way, it should be quite easy to understand the usefulness of these tests when performing the evaluation of a system for biomedical use; however, one thing to take into account is the fact that positive outcomes of *in vitro* genotoxicity tests may not always occur as a consequence of direct reaction of a compound or a metabolite with DNA.[68]

19.5.3.1 *In Vitro* Micronucleus Test

The *in vitro* micronucleus test is usually performed with continuously dividing cells, mostly established cell lines, or human peripheral lymphocytes.[69]

Compared with the chromosomal aberration test (not focused within this chapter), the *in vitro* micronucleus test detects aneugens more reliably, it is faster and easier to perform, and it has more statistical power and the possibility of automation.[61]

Figure 19.2 is a schematic diagram of the main steps performed during the *in vitro* micronucleus test. Briefly, cells are incubated with the dissolved agent — in the case of biodegradable materials, either with leachables from the material or the material itself — for a predetermined period of time (up to 72 hours or less) and the cells are then treated until staining with Giemsa, which will reveal under a microscope the presence/absence of micronucleus.

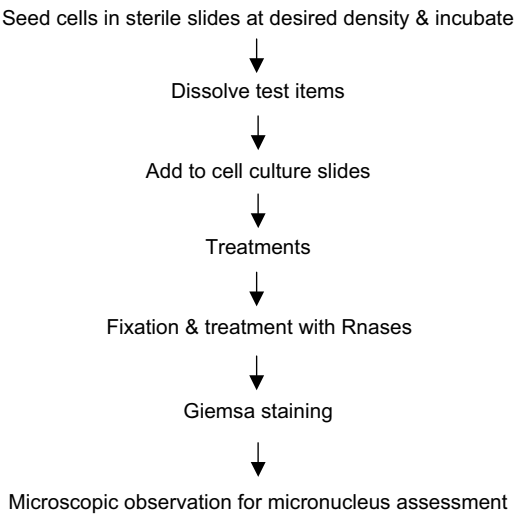

FIGURE 19.2 Schematic diagram of the main steps in the *in vitro* micronucleus test. (Adapted from Pfuhler, S. and Wolf, H.U., *Mutation Res. — Genet. Toxicol. Environ. Mutagenesis*, 514, 133, 2002.)

This test can be performed in a easy way yielding reliable results, and for so, it can be used for biomaterials testing, both as extracts for the biodegradable material as well as the material itself, since the mutagenic potential of a biomaterial is generally assessed on extracts or particles.[67] Leachables are the easiest way to perform this test, but it should also be assessed in the final form of the material since the mutagenic potential is influenced not only by the chemical properties of the biomaterial, but also by the physical properties, especially surface properties: form, size, hardness, surface condition, porosity, surface tension, surface energy, and wettability.[67,71–73] Also, the size of the samples in contact with the cells must be much greater than that of the cells to ensure that the sample size/cell size ratio approximates that *in vivo*.[67] The same principle applies also to the following test, the comet assay or single-cell electrophoresis.

19.5.3.2 Comet Assay or Single-Cell Electrophoresis

Comet assay, also called single-cell gel electrophoresis, was introduced by Ostling and Johanson in 1984[74] and further developed by Singh in 1988.[75] It evaluates damage to genomic DNA in individual cells, caused by genotoxic agents.[76] This assay is frequently used to evaluate the genotoxicity of test substances,[76,77] since it enables sensitive detection of DNA lesions including single strand breaks, alkali-labile sites, and other modifications of the molecules, during the exposure of cells to potent mutagens and ionizing radiation.[78] Because of the simple procedures, high sensitivity, short response time and the requirement of relatively small number of cells and test substances, and the fact that it can be used to analyze any eukaryote cell population,[78–80] it has been a powerful tool for the determination of genotoxicity.[78] The pictures obtained for each cell nucleus comprise a "head" and a "tail," the whole forming a comet-like image. The interpretation of the results is based on the hypothesis that the nuclear DNA damages caused by a noncrosslinking genotoxic agent produce low-molecular-weight DNA strands, either directly through DNA breaks, or indirectly by excision-repair of damaged DNA or formation of alkali-labile sites.[81] These broken DNA strands are released in the course of lysis and unwinding stages of the comet assay process and they produce the comet tail upon electrophoresis, while undamaged high-molecular-weight DNA does not migrate and forms the comet head. On this assumption, the more DNA is damaged by a noncrosslinking genotoxic agent and broken into low-molecular-weight pieces, the bigger the comet tail, and the larger the comet parameters: "tail length," "tail

DNA" (% of DNA in comet tail), and "tail moment" (TM = tail length × tail DNA).[67,82,84] Some authors have suggested that comet pictures might be associated with apoptotic cell nuclei,[83,84] but they limited this interpretation to highly damaged cells (HDC or "ghost cells") that are easily recognizable in situ on the slides. Therefore, interpretation of a comet tail in terms of genotoxicity would be valid, provided that highly damaged cells were disregarded.[83,84] Although varieties of mammalian cells are commonly used in the comet assay, any eukaryotic cells are basically appliable,[85] so in biomaterials testing, one can use the cells typical of the implant site in order to be able to get a more specific response.

In Tice et al. can be found some of the guidelines available for this type of *in vivo* and *in vitro* testings.[85]

19.6 BIOFUNCTIONALITY ASSESSMENT *IN VITRO*

The essential philosophy for studying biofunctionality *in vitro* is that one aims at establishing reproducible and quantifiable assays, which is centered on cells involved in biological parameters relevant to the biomaterial application. Therefore, in the assessment of biofunctionality *in vitro,* it is very important to use the cells that are relevant for the final application of the material, in order to study the more specific aspects of biocompatibility.[11,29,42] This means, for example, that for vascular prosthesis, endothelial cells are appropriate, whereas for studying cell interactions with orthopedic implants, chondrocytes or osteoblasts should be chosen.

It is clear that the relevant parameters in these cases will include some of the functions studied under the heading of cytotoxicity, for example, cell proliferation. However, other cell biological parameters, not yet mentioned, are also of prime importance, such as cell adhesion, cell spreading, and cell biosynthetic function.[11,29,42]

Cell adhesion is probably the single most important aspect of cell interaction with a biomaterial, if we exclude the possibility that components of the material are cytotoxic.[17,29,42,86,87] Cell adhesion is the prerequisite for further cellular functions, such as spreading, proliferation, migration, and biosynthetic activity.[17,29,42,86–91] Therefore, it is a field of obvious interest not just to biologists, but also to material chemists and surface engineers. Surface characteristics of materials, whether their topography, chemistry, surface energy, or wettability, play an essential role in cell adhesion on biomaterials.[88–92] It must be stressed that cell adhesion is desirable for biomaterials that are to be integrated into host tissues, such as orthopedic implants in bone or in vascular prostheses which are to be pre-seeded with the patient's own endothelial cells.[17,42,88,91,93] On the other hand, adhesion is a serious problem for blood-contacting surfaces that are not to be preseeded, such as catheters or sensors.[17,29,42,88,91,93] Quantification can be carried out using image analysis systems at both light and scanning electron microscopic level and thus be used to compare the efficacy of various substrata.[29]

Cell spreading, which involves complex cytoskeleton reorganization, is an essential function of a cell which has become adherent to a surface, and precedes the function of cell proliferation to give a cell-covered surface.[17,42] Cell spreading is thus a parameter of vital importance in studying biomaterials designed to be fully integrated into the host tissues. This parameter can be assessed, for example, by quantitative scanning electron microscopy (SEM).

Already presented as an important parameter concerning cytotoxicity assessment, cell proliferation determination is a central component of studying materials designed to be integrated into host tissues,[17,42] as in the case of osteointegration of a joint prosthesis or dental implant.

Alteration in the biosynthetic activity of a cell may be subtle and may not be of biological significance with reference to biocompatibility. However, there are too few studies of such parameters to permit a critical judgment at present. Nevertheless, it is possible that a biomaterial may not exert a negative influence on cell proliferation *in vitro*, but may significantly reduce production of certain biosynthetic products.[13,94]

19.7 *IN VIVO* TESTING: THE NEXT STEP

After the cytotoxicity screening *in vitro* and the direct contact of the testing systems with cells in order to establish their profile when in contact with the target tissue/organ, it is time to move forward and to take the next step in the assessment of the biocompatibility and functionality of the proposed system: the *in vivo* testing.

Several implantation procedures have been suggested in order to obtain the most adequate system for each type of material for the evaluation of local toxicity. It is important to evidence that besides the materials there are other issues related with the surgical technique that influence quite extensively the host response.[41] Thus, although the most currently used intramuscularly and subcutaneous models represent appropriated choices, new options are always under study trying to reduce as much as possible the interference of other variables apart from the implant.[31,46,95]

Degradable materials, which will stay in the human body for a long-term period, will induce not only local but also systemic effects.[95–97] The degradation products, their concentration, the changes occurred in the materials such as alteration of the shape, roughness as well as, for example, the mechanical irritation of the more degraded materials in the biological tissues, may induce local and remote interactions between material/products and the biological system.[47,98–100] An acute phase pursues the implantation of any material with an extent that depends on several parameters mainly related with the material. If the deleterious action of the materials remains, a chronic response persists, leading in many cases to the failure of the implant.[99,100] Both acute and chronic reactions can persevere for many years. Regardless the tissue in study, the animal should be examined periodically for sinus drainage and sterile sepsis. Besides physical examination, the evaluation of the implant *in vivo* performance can be achieved by biomechanical testing. After sacrificing the animal, shear strength, bending, and tensile tests are commonly performed to compare device characteristics before and after implantation.[101]

The assessment of the type and extension of response that a biomaterial might locally induce is based on the histological analysis of the tissues surrounding the implant. Several factors have been taken in account within that analysis, helping to define the degree of toxicity of the implant. The presence and amount of certain types of cells such as neutrophils, monocytes, macrophages, eosinophils, lymphocytes, fibroblasts, and foreign body giant cells at the tissue–material interface are indicative of the response elicited by the implant.[95,99,100] Immunocytochemistry techniques are often used to identify the cells present in the retrieved implant as well as in the neighborhood tissues and complemented with image analysis systems to quantify the number of cells and their distribution related to the implant.[95,102] Many of these cells, when activated, produce hydrolytic enzymes responsible for the lesion of the tissues; thus, the enzymatic activity in the tissues surrounding the implant can be determined in order to identify the presence or not of the toxic stimulus.[103,104]

Following the implantation of any medical device, the wound healing mechanisms are triggered in response to injury and to the presence of a foreign body.[105,106] Thus, the host generates a response aiming to eliminate the cause of injury and to repair the damaged tissues. The formation of a fibrous capsule is a common occurrence after the implantation of biomaterials, but its formation depends on the severity of the response induced by the implant and consequently from its properties.[47,98–100] Thus, the thickness of the fibrous capsule formed around an implant was also suggested to be a measure of the toxicity of the materials in study.[47,98–100,105,106]

Because of the interconnection between tissues and organs through blood, the lymphatic system, and interstitial tissue fluid, exchange of products takes place between the implantation site and the rest of the body. Remote site effects are slightly neglected within the *in vivo* biocompatibility evaluation of potential biomaterials. Rather than histological analysis, the assessment of systemic effects can be done examining physical symptoms like hypokinesia, dyspnea, diarrhea, cyanosis, tremors, and, the worst scenario, eventually death.[76–78,107]

19.8 CONCLUSIONS

Cytotoxicity evaluation has become a routine in almost all laboratories that work on the development of systems for biomedical applications. Several tests are now routinely established and can be performed at any step of the system development. Standards for testing these materials have been developed and regulation by several organisms does exist. However, one must take into consideration when testing biodegradable materials that present a determined degradation profile that several phenomena such as water uptake and degradation (with leaching of compounds to the medium) can occur during testing. In this way, standardization of cytotoxicity tests for screening biodegradable materials is still a difficulty at present. The transposing of the standardized methods for biodegradable materials testing is used and seems to be adequate to provide information about these materials, not disregarding, however, their properties.

Collecting information from several types of tests — morphological, biochemical, and gene-level — will allow for a full-range characterization of the biodegradable system before further *in vivo* tests are carried out, and in case the material fails in one of these parameters, will lead to its improvement at the synthesis level. These tests can be regarded as the yes/no in the materials evaluation fluxogram. However, no test should be considered solely to make the yes/no decision. Only a combination of tests, giving different answers, can give an accurate final answer to the posed question: Is this material harmless for it to be tested for its biocompatibility and biofunctionally *in vivo*? We must thus have an overall picture of the biomaterial/host interface, or biomaterial–cell interface, especially in the case of *in vitro* studies.

Genotoxicity tests will find increased use in biomaterials testing in future years, as bioengineers aim to get deeper insights on the effects of the materials in the implant site, as well as to correspond to the advances of science in the so-called genetic era, by providing information of the behavior of the materials at the genetic level.

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20 Natural-Origin Degradable Materials: The Barrier or the Passage through the Immune System?

A. P. Marques, J. A. Hunt, and Rui L. Reis

CONTENTS

ABSTRACT

Biodegradable materials perhaps hold much of the future potential for many biomedical applications due to their advantages when compared to nondegradable materials, both in terms of their foreign-body reaction and in their gradual elimination and therefore replacement. The stimulus or source of a tissue reaction is always present with nondegradable biomaterials and might be the queue for chronic inflammation often culminating with failure.

Natural-origin biodegradable materials have emerged, due to their similarities with biological systems, as having additional advantages over synthetic materials. In fact, natural-origin polymeric materials are generally more susceptible to enzymatic degradation and many of the enzymes responsible for that process are present in the human body. The degradation products are often

comprised of molecules that can be incorporated in normal metabolic processes. In addition, it would be expected that any other molecules/fragments resulting from the degradation of natural-origin implants, because of their nature, could be easily eliminated from the surrounding tissues, thus minimizing inflammation. However, several other aspects should be analyzed and taken into account when considering foreign-body reactions to biomaterials.

This chapter will give an overview of the biological elements involved in the inflammatory response to biomaterials, as well as introduce the reader to the significant parameters of materials that might enable modulation and control of biological reactions with particular reference to degradable natural-origin materials.

20.1 INTRODUCTION

A large number of synthetic materials are proposed or used currently in a wide range of biomedical applications.[1–5] Many are successful, but few are considered ideal and most materials can be recognized as foreign by the host and prompt a tissue response.[6–10] In general, they do not exhibit comparable physical, chemical, or biological properties to natural tissues, and ultimately, these devices can lead to chronic inflammation and foreign-body reactions.[7,8,10–12]

Therefore, the search for improved material's performance, which has a fundamental synergy with living systems, constitutes one of the major challenges for materials scientists and biologists. Natural-origin materials have been presented as potential solutions for the lack of biocompatibility and immunocompatibility of currently used devices.[13–22] Nevertheless, a compromise between suitable mechanical properties for the proposed applications in order to guarantee existing functions, and the physical and chemical characteristics of the materials has yet to be achieved. Thus, one of the research goals within the biomaterials field is to create new devices that can prevent foreign-body reactions and promote normal wound healing.

From the biological perspective, the surface elements that interact with materials, such as proteins and cells, represent the key aspects in the development of successful biomaterials. To date, a complete understanding of the biological responses to implanted biomaterials is still missing. The mechanisms of how a body reacts to implants over the course of time by inflammation, wound healing, and the foreign-body response is not fully understood. Leukocytes have been identified as the main cell types responsible for the adverse reactions implicated in inflammation.[11,23,24] Severe and persistent leukocyte activation may lead to compromising alterations in the function of an implant and eventually to the failure of the device. In addition, there is some controversy about which properties of the surface of materials stimulate particular cell/tissue reactions. It has been hypothesized that not only the wettability and surface charge of the surface of the materials, but also the presence of certain functional groups have importance for the adhesion and activation of immunological cells *in vitro*.[25–32] Furthermore, the degradation rate and mechanisms of biodegradable devices can also modulate[33–36] and might allow control of tissue responses *in vivo*.

20.2 IMMUNE SYSTEM

Immunity refers to the ability of an organism to resist disease by identifying and destroying foreign substances or organisms.[37,38] Cells and molecules involved in such mechanisms constitute the immune system and the response resulting from the introduction of a foreign agent is known as the immune response. However, not all immune responses occur to protect the host from disease; there are situations, although not the majority, when sickness is caused by an immune reaction.[39–43] These are, for example, allergic reactions,[44] which occur due to the presence of external stimuli, or autoimmune diseases such as multiple sclerosis[42] or rheumatoid arthritis,[41,43] where an individual reacts against their own tissues. The implantation of any medical device can be considered an

external invading element that might induce an immune response, mostly but not exclusively, dependent on the properties of the device.[25–27,32,45–47]

Central organs of the immune system include the bone marrow and thymus, which are involved in generating precursor lymphocytes rather than immune responses.[48,49] In addition to lymphocytes, monocytes and granulocytes also derive from precursor stem cells in the bone marrow.[49] The lymph nodes and spleen are known as peripheral organs and have as their main role the optimization of interactions between antigen presenting cells (APC) and T and B lymphocytes.[48]

20.2.1 Inflammatory Cells

20.2.1.1 Lymphocytes

Lymphocytes have receptors for antigen and confer specificity to an immune response. These cells express receptors with varying affinity for the antigen in question; thus, during lymphocyte development, the cell with the highest affinity for the most abundant antigen will have a growth advantage and will preferentially generate progeny of itself.[48]

There are two types of lymphocytes, B and T[49]; B cells have their origin in the bone marrow of adult mammals, whereas T lymphocytes undergo further maturation in the thymus. B lymphocytes migrate directly from marrow to peripheral lymphoid tissue, producing antibodies and some soluble mediators called cytokines.[50] On the contrary, T lymphocytes do not produce antibody molecules, but have surface receptors structurally related to immunoglobulins (Ig).[51]

Once released from the bone marrow and thymus, lymphocytes begin to populate the whole lymphoid system. The higher concentration and the degree of accessibility of these cells enables a rapid response to infectious agents.

T cells see antigen by recognizing peptide fragments complexed with surface Major Histocompatibility Complex (MHC) glycoproteins on neighboring cells. The cell surface glycoproteins, encoded by genes in the MHC allele, bind fragments of antigen after they have been subjected to antigen processing.[52]

There are two subsets of T cell divided according to their function: T helper (Th) and T cytotoxic (Tc). Both are involved in cytokine production, but they also have individual actions, respectively helping B cells and T cytotoxic cells in the lysis of infected and tumor cells.[49] Surface proteins, expressed by the different cells of the immune system, have been given standardized names, characterized by the initials CD (Cluster Designation) and a number. CD4 and CD8 are specific for the two T cell subsets, but the CD markers can be specific for individual populations of cells, or particular phases of cellular differentiation or activation. Th cells express CD4 and present antigens in association with MHC class II molecules, while Tc lymphocytes express CD8 and present antigen using MHC class I proteins.[53]

B cells use a different mechanism of antigen presentation; after binding to a cell surface antibody, their specific antigen is internalized, partially degraded, and presented to Th cells in association to MHCII molecules.[54]

Natural killer (NK) cells are large granular lymphocytes that are cytotoxic in the absence of prior stimulation. NK cells represent a first line of defense to infections, tumor growth, and other pathogenic alterations of tissue homoeostasis, possessing receptor molecules which allow them to detect some infected host cells, including tumor cells, virus, or intracellular bacteria-infected cells.[49] NK cells do not express antibodies or T cell receptors at their cell surface, but produce cytokines and express receptors for immunoglobulins.[55]

20.2.1.2 Mononuclear Phagocytes

Phagocytic cells are critical in the defense against bacterial and simple eukaryotic pathogens.[37] Mononuclear phagocytes, in particular monocytes/macrophages, can recognize bacterial and yeast cell walls through broadly specific receptors, usually for carbohydrate structures, being able to take

them up by phagocytosis.[56] Besides monocytes/macrophages, mononuclear phagocytic cells also include microglial cells in the central nervous system, endothelial cells of vascular sinusoids, and reticular cells of lymphoid organs, which take up large particulate antigens, pieces of tissue, senescent cells, and bacteria.[49]

These cells have important properties; they express a myeloid receptor (CD14) which serves as a recognition molecule for a wide variety of bacterial envelope molecules, such as lipopolysaccharides (LPS), which after interaction lead to macrophage activation.[57] Mononuclear phagocytes can act as APCs for T cells and at the same time can be activated by T-cell-derived cytokines, leading to increased phagocytosis and microbicidal activity (increased activity of degradative enzymes, prostaglandins, nitrogen, and oxygen free radical production).[58] Furthermore, these cytokines also increase the antigen presenting activity of macrophages, which, in turn, are able to present antigen to T cells. This cycle will continue as a positive feedback loop until the antigen is eliminated.[59]

Mononuclear phagocytes express receptors for antibodies and complement, which means that they bind immune complexes, especially if the antibody involved has complement components bound to it, in which case cells endocytose/phagocytose these rapidly.[60]

20.2.1.3 Dendritic Cells

Within the group of dendritic cells, there are two cell types with similar names but different functions. Cells of the dendritic cell (DC) lineage are bone marrow derived, but they are also present in the skin where they are known as Langerhans cells (LC).[49] These cells efficiently process antigen, but cannot present it to T cells. LCs have been shown to pick up antigen in skin and carry it via afferent lymphatic vessels to lymph nodes. Here, the cells known as tissue dendritic cells or interdigitating cells, may efficiently present antigen if they encounter the right T cell. In fact, these are the most efficient APC, since far fewer DCs are required to initiate an immune response than any other APC.[61]

20.2.1.4 Granulocytes

There are three types of granulocytes[62]: neutrophils, eosinophils, and basophils. Neutrophils, also known as polymorphonuclear leukocytes, express receptors for immunoglobulin and complement and are involved in the acute inflammatory response. Eosinophils carry receptors for immunoglobulin E (IgE), are involved in the destruction of IgE-coated parasites, and contribute to the response to allergens.[63] Basophils are the circulating counterpart of tissue mast cells. They express high affinity receptors for IgE and are stimulated to secrete the chemicals responsible for immediate hypersensitivity following antigen-induced aggregation of these receptors.[63]

20.2.2 Immune Responses

Immune responses can be distinguished based on different approaches. It is possible to make a distinction between innate and adaptive immunity, respectively, as the capacity to respond to foreign agent instinctively or in a specific manner. The innate or natural response is present in neonatal animals, while in the case of adaptive response, the immune system requires preactivation toward the foreign agent. Natural immunity depends on a variety of immunological effector mechanisms which are neither specific nor improved by repeated encounters.[38] Among others, its components are the complement system, acute phase proteins, and interferons. In turn, the adaptive immunity increases in effectiveness and strength each time the host contacts the invaders.[37,38] This constitutes a useful evolutionary adaptation because it improves the efficacy of the innate immune response by focusing the response to the site of invasion/infection as well as providing additional effector mechanisms that are unique to lymphocytes.[37,64] The difference between innate and acquired

immunity lies in the antigen specificity of lymphocytes.[37] This property is conferred upon lymphocytes by the expression of cell surface receptors that recognize discrete parts of the antigen.

From a different perspective, it is possible to organize the immune response in a dual system known as cell-mediated immunity and humoral immunity. Both systems are adaptive and respond specifically to most foreign substances although, depending on the antigen, one immune response generally is favored over the other.[65] Lymphocytes are involved in both types of reactions. T cells are responsible for the cellular immunity because they are directly involved in the response, and B lymphocytes are implicated in the humoral response reacting with the antigen and producing antigen-specific antibodies.[37,38] An antigen is then defined as any substance that can bind to a specific antibody and comprises an enormous range of substances from simple chemicals, sugars, and small peptides to complex protein complexes such as viruses.[37]

During an immune response, a complex lattice of interlinked antigens and antibodies, known as an immune complex, will present an array of constant regions, which can activate cells through the binding of their immunoglobulin receptors. Antibodies can act in different ways, by blocking the biological activity of their target molecule — e.g., an enzyme binding to its receptor (neutralization), interacting with special receptors on various cells, including macrophages, neutrophils, basophils, and mast cells, allowing them to "recognize" and respond to the antigen (opsonization), and causing direct lysis by complement, which also enhances phagocytosis (complement activation).[37]

20.3 FOREIGN-BODY REACTION TO IMPLANTED MATERIALS

The implantation of a biomaterial initiates a cascade of events, generally described as a foreign-body reaction, which varies in time and in the inflammatory mediators involved.[26,45] The duration and intensity of the response depends on several elements including the extent of the injury caused by the implantation procedure, factors related with the host,[66,67] and numerous properties of the implant such as chemical composition, surface free energy, surface charge, roughness, size, and shape.[26,28–32,68]

The significant properties of biomaterials have been the focus of much research, probably because they constitute the controllable variables[69–73] in the development of potential biomaterials. The emergence of biodegradable materials introduced more complexity to the biological response.[74] Together with the foreign-body reaction, the material is degrading, which may lead to changes in shape, surface roughness, release of degradation products,[73,75,76] formation of particulates,[77,78] and hence, from the host perspective, potentially new elements to respond to.

Histological analysis enables the degree and extent of the foreign body reaction to be determined. Fibrous capsule formation around biomaterial implants is considered a normal response.[79] Some authors[80] reported that the thickness of the capsule between 20 and 30 μm would be the ideal situation for biocompatibility. Others[81,82] suggested that materials that induce a thin-walled capsule containing quiescent fibroblasts and a small number of macrophages were considered biocompatible. Imai et al.[83] reported that the threshold capsule thickness should not exceed 200–250 μm for an implanted hydrogel. In addition, Marchant et al.[84] have observed that the total collagen content was about 0.23 to 0.27 mg/mg tissue dry weight in 21 days postimplantation of a hydrogel.

However, it must be clarified that the biocompatibility of any material includes biofunctionality.[85] Therefore, even if by inducing an inflammatory response the function for which the device was designed is not compromised and there are no complications associated with the implantation of the device, it must be seen as biocompatible. For example, because collagenous encapsulation may impede the biofunctionality of implantable drug delivery systems, in this particular application, the objectives are a diminution of the capsular thickness and an enhancement of angiogenesis around such devices.[86] Contrarily, in the case of devices where diffusion is important such as sustained-release systems[87] and implantable bioartificial organs,[88] encapsulation by a fibrotic capsule can be a considerable impediment to device function.[89]

20.3.1 Wound Healing

Wound healing is a very complex process that emerges after tissue injury with the aim to seal and make stable an area of damage.[90] This response involves a series of distinct stages which can overlap in terms of time.[91] Dysfunctions in any of these phases can induce disorders in the healing process; thus, the nature and severity of an injury are dependent on the normal course of the reparative process.[92] If the injury is minimal, the repair process takes place in a short time, since the complete repair of the tissues might be accomplished by the regeneration of parenchymal cells only.[93] However, with more extensive wounds, the inflammation is more severe and the repair process involves scar tissue formation and possibly the loss of functions.[92] It is under this situation, where the inflammatory reaction is more complex, due to the persistent presence of the causing agent and with few mechanisms to deal with it, that the implant devices might be considered.[93]

The phases of normal wound healing follow an orderly sequence of events that are characterized and regulated by the chronologic appearance of a number of different cell types.[91] Once these cells undergo activation, i.e., phenotypic alterations of cellular, biochemical, and functional properties, thrombin inside the plasma clot induces platelets to degranulate releasing the contents of their alpha-granules, which, in turn, leads to the expression of new cell surface antigens, increased cytotoxicity, and increased production and release of cytokines.[94,95] These, together with other factors, activate cells that then mediate subsequent phases of the wound-healing process.

The first cells arriving to the site of injury are neutrophils, reaching a peak after 24 hours, being removed by tissue macrophages when they are no longer needed.[26] Monocytes appear approximately 24 hours after injury, reaching a maximum number 48 hours postinjury and fibroblasts migrate into the wound from day 3 onward. Since monocytes mature into macrophages, they can be considered an essential source of cytokines, which then drive repair processes. A variety of chemokines are also responsible for the spatial and temporal infiltration of leukocyte subsets and therefore control the integration of inflammatory and reparative processes during wound repair.[45]

20.3.2 Acute Inflammation

Acute inflammation is the generic term used to label the complex process of endocrine and metabolic or neurological changes observed in an organism, either locally or systemically, a short time after injuries, infections, and immunological and inflammatory reactions.[96] Thus, any type of disorder or tissue injury inevitably involves an acute phase reaction.

Many infections, especially where small wounds are the route of entry, are eliminated by the combination of complement and recruitment of phagocytes, which flow from the acute inflammatory response.[97] In fact, the acute reaction is initiated and mediated by many cytokines and by different types of cells, such as PMN, fibroblasts, endothelial cells, monocytes, and lymphocytes.[62] The development and control of the reaction occurs through the interaction of the numerous cascades of cytokines within the different tissues.

Locally, the acute phase is characterized by an increase in blood flow to the site of injury, enhanced vascular permeability, and a well-organized and directional influx and selective accumulation of different leukocytes from the peripheral blood at the site of injury/implantation.[62,98] In particular, the number of circulating neutrophils and monocytes increases. Neutrophils are highly destructive cells capable of mounting a rapid, nonspecific phagocytic response, while monocytes mature into macrophages, which are responsible for the removal of necrotic tissue, the phagocytosis of foreign materials, and the release of growth factors.[96]

A short time after implantation, any implanted device has many proteins adsorbed onto its surface.[99–102] Some of these proteins such as IgG, fibronectin, and complement C3 fragments work as opsonins.[100,102] Neutrophils and macrophages bind to the implant surface through receptors for those proteins, which results in cell attachment, activation, secretion of reactive oxygen species, and the release of proteolytic enzymes.[45,103] These cells will attempt to phagocytose implants. If

successful, implant particulates may be a subsequent product. If the implant is too large, frustrated phagocytosis will occur. Either process can release these powerful destructive species, stimulating further inflammation.[98,104–106] The presence of a device can induce a massive and excessive activation of leukocytes, sustaining an inflammatory response.

In addition to local activities, some systemic effects are typical of this type of reaction. For instance, fever is a very common sign, but also cellular and biochemical changes occur in the liver with the synthesis of the so-called acute phase proteins.[97]

20.3.3 Chronic Inflammation

Chronic inflammation is a process that takes place when an immune stimulus persists for a prolonged period of time, beyond that in which the immune system would normally have eliminated the antigen.[37]

Following neutrophils, other cell types like monocyte/macrophages and particularly lymphocytes (specific subsets of T cells and B cells) and plasma cells migrate to the site of injury.[26,107] These cells involve antigen-specific and more tightly regulated immune responses, and once activated, they also produce protective and inflammatory molecules. Another important characteristic of chronic inflammation is the development of blood vessels[108,109] and the production of connective tissue.[93]

Once again, the implant is a determinant for the progress and resolution of the reaction. If the foreign body continues to resist, the host defense becomes more aggressive and a special type of inflammatory tissue is formed. Macrophages fuse forming foreign-body giant cells (FBGC), fibroblasts proliferate, collagen and proteoglycans are synthesized, and angiogenesis takes place to form the so-called granulation tissue.[93]

20.3.4 Reparative Phase

The first step of the reparative phase involves the activation of the intrinsic part of the blood coagulation cascade. This starts when the injury takes place due to ruptures of the blood vessels which instigate the contact of plasma with tissue and basal membranes of cells and the exposure of subendothelial collagen to platelets.[45] The formation of a fibrin gel serves to fix plasma proteins and blood cells, which leads to hemostasis, and acts as a scaffolding matrix that can be populated subsequently by inflammatory cells such as neutrophils, monocytes, and macrophages as well as fibroblasts and endothelial cells. Thus, inadequate clot formation is associated with abnormal wound healing.[93]

Fibroblasts and endothelial cells are the primary proliferating cells arriving, as the number of neutrophils decreases and the number of macrophages and fibroblasts in the wound area increases.[26,45,107] Fibroblasts replicate in response to cytokines and growth factors present in the surrounding tissues which were released during the earlier phases of wound healing and stored in the fibrin clot, which is invaded by these cells. Fibroblasts deposit the collagen that forms part of the substance of granulation tissue.[26,110]

The formation of new blood vessels is initiated by endothelial cell migration and into the healing wound. The formation of new blood vessels within the wounded area is essential for normal fibroblast and leukocyte function, thus the process is maintained as long as required by various angiogenesis factors.[107]

The final phase is characterized by the gradual replacement of granulation tissue by fibrous connective tissue, a process that also requires locally acting cytokines. Collagen is synthesized and the closure of the wound and formation of the scar is accompanied by a decrease in cellularity, including the disappearance of typical myofibroblasts.[93]

The presence of an implant also plays a role in the disruption of the normal sequence of events of the reparative phase. Angiogenesis is restricted, due to the inability of capillaries to migrate

through the device, as well as the pattern of collagen deposition being different, either would most likely result in slowing down the repair process and prolonging inflammation.[93] Furthermore, the presence of considerable amounts of granulation tissue may be responsible for an adjustment of the mechanisms of repair and their replacement by extensive scar tissue lacking specialized functions, instead of the ideally minimal fibrous encapsulation of the implant.[79,111]

20.4 HYPERSENSITIVITY TO METALS

As it was mentioned before, besides helpful immune reactions, there is another type of immune response that has harmful consequences for the host, normally described as hypersensitivity.[63] So far, hypersensitivity reactions were subdivided into four types, three mediated by antibodies and one mediated by T cells.[37,63,112]

Type I hypersensitivity reactions are the most currently described because these correspond to the rapid allergic reaction. The symptoms resulting from exposure depend on the site of contact. Mast cells and basophils have a high density of antigen-specific receptors and become activated after the contact between those receptors and the allergen. This induces rapid cell degranulation, releasing primary inflammatory mediators stored in the granules. The mediators initiate a sequence of events characteristic of acute inflammation. Furthermore, cell activation also induces the production of secondary mediators such as prostaglandins, leukotrienes, cytokines, and enzymes.[37,63]

The second class of destructive reactions, type II, is caused by specific antibody binding to cells or tissue antigens. The antibodies directly or indirectly cause cell destruction, through the recruitment of complement. Usually the target cells are foreign to the host, but they might not be, as in the case of autoimmune diseases, which means that this type of hypersensitivity reaction is only found in blood transfusion recipients and patients with certain autoimmune diseases.

The type III hypersensitivity reaction has much in common with the type I response. It is also mediated by antigen–antibody immune complexes although with a different antibody and consequently without the involvement of mast cells.[63] These complexes deposited at various sites triggering neutrophils to release their granule contents with consequent damage to the surrounding tissues.

Finally, the type IV reaction, also called delayed type hypersensitivity, which is the only class of hypersensitive reactions to be triggered by antigen-specific T cells. In this situation, the antigen is picked up by an antigen presenting cell, typically dendritic cells, processed and presented in association with MHCII molecules to Th lymphocytes. Therefore, T cells become activated, producing cytokines such as chemokines (chemoattractant for macrophages, other T cells, and to a lesser extent, neutrophils) as well as Tumor Necrosis Factor β (TNF-β)) and Interferon γ (IFN-γ)). The consequences are a cellular infiltrate in which mononuclear cells tend to predominate.

One of the major problems of the currently used metallic biomaterials is organometallic complexes, possibly being the result of the reaction of the corrosion products, which are considered to be antigens/allergens[113] and the metallic ions with the proteins of the host.[114,115] Metals accepted as sensitizers are nickel,[116,117] beryllium,[118] cobalt,[115,119] and chromium,[115,120] and occasional responses have been reported to tantalum,[121] titanium,[122] and vanadium.[123] The most common metal sensitizer in humans is nickel, followed by cobalt and chromium.[116,117,124] Although little is known about the mechanisms of interaction and the dynamics of the metallic products *in vivo*, there are many immunologic type responses reported and associated with cardiovascular,[125,126] orthopedic,[116,122,123] plastic surgical,[127] and dental implants.[128,129] These reactions are generally associated with the hypersensitivity type IV response,[117,118,123,124] most likely mediated by wear debris products and leading to specific responses such as severe dermatitis, urticaria, and vasculitis.[117,121,124] Furthermore, other effects such as metabolic alterations, changes in host/invader interactions, formation of lymphocyte toxins, and initiation or promotion of chemical carcinogenesis may come together with the direct immune response.[79,107,130,131]

20.5 IMMUNOREACTIVITY TO NATURAL-ORIGIN VERSUS SYNTHETIC BIODEGRADABLE SYSTEMS

Currently used biodegradable materials for biomedical applications are mainly synthetic.[1–5,71,132] Natural-origin biodegradable polymers such as polypeptides,[15,18,19,21] polysaccharides,[13,14,17,20,133] and bacterial polyesters[22,134] have been proposed as an alternative for a wide range of those applications.

Unpredictable adverse reactions to some commonly used traditional implants have been reported during the years.[6–10] Nevertheless, poly(lactide acid) and its derivatives are the most commonly used synthetic biodegradable materials, and they are widely accepted as biocompatible.[135–138] In the first phases of polylactide degradation *in vivo*, only hydrolysis takes place.[139] The final products of the disintegration have to be removed by cells, which would normally be involved in inflammation. In fact, both clinical applications[7–9] and animal studies[34,140,141] have suggested that degradation products directly and indirectly affect tissue remodeling, respectively, by interaction with the cells responsible for the formation of *de novo* tissue and through the induction of inflammatory cytokines released by activated macrophages. Therefore, the influence of the degradation time was addressed with long-term *in vivo* studies, but the results were not conclusive, demonstrating acute to mild inflammation.[77,141,142] Long-term evaluation of implanted poly-*L*-lactic acid (PLLA) screws and plates[9] showed that some patients presented intermittent swelling at the site of implantation which was classified, after investigation of the nature of the tissue explanted, as a nonspecific foreign-body reaction to the degraded PLLA material. Furthermore, remnants of degraded PLLA were surrounded by a dense fibrous capsule and internalization of crystal-like PLLA material in the cytoplasm of various cells was also noted.[9] A comparable result was observed in another study[8] which applied polyglycolic acid (PGA) and lactide–glycolide copolymers. Only 7.9% of patients developed complications, identified as a nonbacterial inflammatory response which was typical of a nonspecific foreign-body reaction. A more serious complication was detected 4 months after osteosynthesis of medial malleolar fractures with PGA implants.[11] Lymphocytes were the main type of cell found in the retrieved cell suspension with a low number of mononuclear phagocytes, which suggested a lymphocyte-mediated immunological reaction against the implant.

Some studies comparing nondegradable and biodegradable materials[23,77,143–145] reported a less favorable host reaction when degradable materials were used, although the differences become less notorious for longer times of implantation.[77] Furthermore, the expression of MHC II has been reported in response to several materials, independently of being biodegradable, such as PLLA,[145] hydroxyapatite-coated prostheses,[146] polymethylsiloxane,[147] and titanium.[148]

Many uncertainties are still present, but several factors have been implicated in the occurrence and intensity of an inflammatory response against biodegradable implants. The difference in the rate of degradation and subsequently the difference in the release of the degradation products, such as monomers, oligomers, and finally fragments, have been considered to be of major importance. The issue is that the rate of degradation might be too fast, allowing the inflammation process to take over, thus compromising the role of the device.[33,149] The inflammatory cell reaction has been reported[23,34,36] to be more intense for polymers that deteriorate rapidly. However, a too slow or hardly detectable degradation can also be undesired for some applications such as the use of biodegradables to support osteosynthesis. Pistner et al.[150] showed that although the polylactides presented moderate inflammation, characterized by macrophages and giant cells only during the first few weeks of implantation, some of those polymers did not present an adequate degradation rate for the proposed function.

Degradable glasses were found to stimulate an inflammatory response in soft tissue,[12] which was clearly associated with its degradation rate. No chronic inflammation was observed after the implantation of a slowly degrading glass, while the fastest degradation rate leads to tissue damage and necrosis. The high numbers of mast cells in fibrous tissue at an implant site was suggested to be linked with allergic reactions to the presence of glass.

The degradation process seems in turn to be influenced/controlled by other variables. It has been suggested that geometry and dimension of an implant influence biodegradation. Some works showed that implantation of a porous material does not induce a dense fibrous capsule[151,152] and enable a better vascular invasion of the polymer bulk.[86,153,154]

Moreover, particle size was suggested as an issue in the different tissue reactions. This is consistent with the fact that macrophages will tend to digest smaller particles[155] and form multinuclear giant cells to surround larger objects,[156] although the differences in duration of their response also may have to be partly related with the material properties.[156] Several studies[24,157–161] have highlighted other extrinsic and intrinsic factors with influence in the biodegradation of biomaterials and consequently on the tissue response. Among them, it is possible to refer to pH[157] and the type of electrolytes of the degradation media,[24,159] the external stress/strain applied,[159] the temperature,[158,160] and free radicals.[24,161] It has also been suggested that the results of biocompatibility studies must be aware of species differences[162,163] and the site of implantation[164,165] of the models in use.

With the emergence of natural-origin materials, the research approach assumed that a combination of synthetic macromolecules with natural macromolecules might yield composites[72,166–171] whose properties would combine the advantages of both materials, respectively, mechanical stability and biological acceptability. In fact, collagen–poly(HEMA) hydrogels were found to be well tolerated when subcutaneously implanted in rats,[166] overcoming the problem of enzymatic resistance and inertness. Histopathological data indicated that the tissue reaction at the implant site progressed from an initial acute inflammatory response characterized by the presence of eosinophils and polymorphs to a chronic response marked by few macrophages, foreign-body giant cells, and fibroblasts. An artificial connective tissue matrix constructed from the association between elastin or elastin-solubilized peptides and type I + III collagens was investigated.[172] Biocompatibility studies in rabbits indicated that the material is totally integrated into the surrounding tissue after a moderate inflammatory response.[172]

Grimandi et al.[173] proposed to develop an injectable bone substitute for percutaneous orthopedic surgery composed of methylhydroxypropylcellulose and a biphasic calcium phosphate, which showed preliminary encouraging results *in vivo*. The inflammatory process was shown to be resolved after 15 days of subcutaneous implantation, and at the same time, a decrease of calcium phosphate granules and extracellular matrix formation was observed.

A hylan gel, an insoluble form of hyaluronic acid, was also presented[174] as an effective alternative for soft-tissue augmentation, due to its unique properties and its capacity of not eliciting inflammatory reactions.

Natural materials have been proposed for biomedical applications mainly due to their similarities with the biological components of the host. However, there are some issues which should be considered[154,175,176]; animal-origin biomaterials may have a further problem when compared with synthetic materials. Specific immune stimulatory effects leading to humoral immune responses have been reported[154,175] and associated with contaminating proteins from the source organism of the implanted material which accentuates the need to use highly purified grades of natural-origin materials. With the growing use of collagen-based biomaterials, questions have been raised regarding the immunogenicity of this protein in humans.[177] Furthermore, most natural-origin polymers are generally degraded in biological systems by hydrolysis followed by oxidation or enzymatically.[159,178,179] Contrarily, the majority of biodegradable synthetic polymers are not subjected to the action of enzymes and are hydrolyzed by the action of water or serum.[159]

Natural-origin materials are subjected to strong physiological reactions and their degradation *in vivo* will depend on the enzyme concentration within each living tissue. The enzyme concentration or other physiological conditions can determine the degradation rate; however, conclusions in terms of inflammation are not straightforward. Alginates, for example, are not subjected to enzymatic degradation when implanted in mammals, therefore presenting a limited and uncontrolled hydrolytic degradation *in vivo*.[180] On the other hand, chitosan is highly sensitive to enzymatic degradation in particular to lysozyme action,[181,182] a neutrophilic enzyme released to the tissues after activation.

Chitosan was found to be uniformly degraded *in vitro* by enzymatic degradation,[182] but the extensive *in vivo* cellular response suggested that other degradation mechanisms as well as other factors were involved in that response.[182,183] However, other works with chitosan materials[184–186] revealed a mild tissue reaction with vascularization of the implant, involving neutrophils which resolved with increasing implantation time and changed to a fibroblastic population.

Starch-based materials, other natural-origin polymers, have been shown to be degraded by α-amylase and phagocytosed by macrophages[163,187] showing an excellent tissue reaction when implanted in both rats and mice.[163,188] In works by other groups,[189,190] starch-based materials implanted in rabbits and goats performed well and without adverse reactions. The host response to crosslinked high-amylose starch (Contramid®) was found to be in accordance with the main phases of the inflammatory and foreign-body responses to injuries caused by implanted devices.[26,93,98,152] After 4 months, only a small residual scar was apparent macroscopically and it was even related to a less severe early reaction than a skin incision and closure with suture material sham.[163]

Kohane et al.[156,191] proposed novel lipid-protein-sugar particles as drug delivery systems. It was suggested that the particles would be biocompatible due to the natural compounds[156] together with expected faster degradation rates when compared with other polymeric delivery systems.[136,192] Their residue would not be expected to remain within the tissues, inducing the formation of FBGC. In fact, the evaluation of the *in vivo* tissue response of two tyrosine-derived polymers (poly[DTE carbonate], poly[DTE adipate]) in comparison to PLLA showed that the response to PLLA fluctuated as a function of the degree of degradation and that the natural-origin materials did not exhibit significant inflammation.[193] In addition, they degraded faster, probably due to enzymatic action, allowing tissue ingrowth into the implant while no comparative ingrowth of tissue was seen for PLLA.

Polyhydroxybutyrate (PHB), a polymer made by microorganisms under conditions of nitrogen deficiency, has been proposed as a biodegradable implant material on the basis of its known degradation characteristics in certain biological environments.[134] Monofilaments of PHB have been studied *in vitro* and *in vivo* up to 180 days.[194] It was possible to demonstrate that although these materials present a very slow degradation rate, its exposure to gamma radiation increases its susceptibility to degrade. Another work[195] showed that the slow degradation rate can be modified by using a filler reinforcement and by changing the initial molecular weight of the polymer. This approach allows these materials to induce a bone tissue adaptation response with no evidence of an undesirable chronic inflammatory response after implantation up to 12 months.[195]

The degradation of collagen-based materials is usually controlled using chemical crosslinkers such as glutaraldehyde, formaldehyde, or hexamethylene diisothiocyanate, which also involve the potential toxicity of unreacted chemicals gradually leached, contributing to increase the duration and intensity of the inflammatory response.[196] Due to the toxicity problem of the crosslinking agents, other less toxic and more biocompatible[197–199] materials are being the aim of several research works. Some of them have been found to elicit significantly less inflammation[199] than the control collagen fibers crosslinked with the currently used agents.

The subcutaneous implantation of glutaraldehyde-crosslinked dermal sheep collagen (GDSC) showed an increase in infiltration of neutrophils with a deviant morphology when comparing with hexamethylene diisothiocyanate-crosslinked dermal sheep collagen (HDSC).[200] Furthermore, a high incidence of calcification was observed, which may explain the minor ingrowth of giant cells and fibroblasts, and the poor formation of new rat collagen. Acyl azide–crosslinked dermal sheep collagen (AaDSC) first induced an increased infiltration of macrophages, and then of giant cells, both with high lipid formation. Other authors[201] defend that collagen crosslinking preserves material integrity for a longer time, additionally decreasing tissue responses at late time intervals, probably by reducing matrix biodegradability and antigenicity. Dermal sheep collagen (DSC) materials crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and *N*-hydroxysuccinimide (ENDSC) induced the same mild cellular reaction as HDSC, whereas, similar to AaDSC, the degradation rate was slow and an optimal rat collagen matrix was formed.[201]

Following implantation, blood proteins immediately adsorb to the surface of the implants. Physicochemical properties of the implant therefore regulate that adsorption and consequently cell adhesion. Previously, implants with increased water and carboxylic group content have been shown to inhibit macrophage adhesion and multinucleation, probably because hydrophobic interactions participate in cell–matrix interactions.[202,203] Additionally, an inhibitory effect of carboxyl groups on macrophage spreading has been reported.[204] Glycosaminoglycans (GAGs) are highly negatively charged polysaccharides due to the presence of sulfate and carboxylic groups, which may contribute to the reduced foreign-body reaction in the presence of those materials.[205]

Anionic collagen membranes, negatively charged at physiological pH, presented advantages over other collagen materials also crosslinked with glutaraldehyde.[206] Besides the controlled biodegradability, the inflammatory response progressed from an intense acute response after 3 days of implantation (polymorphonuclear cells and lymphocytes) to a moderate reaction characterized by the presence of mononuclear cells with low activity after 60 days.[206]

In recent years, hyaluronic acid has been used for many clinical purposes[207–209] and the search for new derivates continues. The esterification of hyaluronic acid with alcohols leads to the preparation of semisynthetic insoluble polymers (HYAFF) with different physicochemical properties allowing modulation of its biological properties.[207] In fact, fibronectin, collagen, and fibrin were shown to react readily with these polymers *in vitro*,[210] therefore modulating the cell response. *In vivo* tests in rats were performed[207] with several HYAFF esters, which induced different tissue reactions. Cell exudates revealed a poor polymorphonuclear infiltration for all the materials, but the most hydrophilic material showed an almost exclusive monocyte and macrophage population around it that was responsible for its resorption.

In general, foreign-body reactions toward implants, manifested by the presence of giant cells, are frequently observed,[26,110,200] and if the duration and extension of the reaction does not compromise the role of the device, they can be considered harmless. However, specific immune reactions have been observed with some natural-origin materials. Calcium alginate dressings have beneficial effects on wound healing by providing a moist wound environment, favorable for cell regeneration.[211] The commercially available dressings may enhance wound healing through mechanisms that induce the stimulation of monocytes to produce pro-inflammatory cytokines.[212] Although this may be advantageous due to the stimulation of cellular activity at the chronic wound site and thereby enhance the healing process,[213] it can promote unresolved chronic foreign-body reactions.[214] Novel freeze-dried alginate gel dressing low in calcium ions demonstrated improvements, showing reduced cytotoxicity and significantly reduced foreign-body reaction when compared with commercially available calcium alginate dressings.[215,216]

Though the numerous collagen-based devices are reliable and effective, it has long been recognized that some subjects may develop an immunity to the collagen or other components in them. Some studies demonstrated that bovine collagen implants have weak antigenic activity and that the immune responses to this implant are typically localized reactions.[177,217] However, early clinical studies with injectable collagens[218–220] verified that approximately 3% of the population develops hypersensitivity reactions. Lymphocytes were observed at the implantation site of collagenous matrices.[205,221,222] Studies with T cell-deficient rats[221] showed that T cells play a major role in the formation of giant cells and the phagocytosing activity of macrophages and giant cells during the tissue response to HDSC. This means that the tissue reaction to biomaterials might be modulated by controlling T cell activation in the case of unwanted or secondary burst reactions, or in the case of too-fast degradation of biomaterials. Hung et al.[223] showed that sacchachitin, a chitin derivative, accelerates the wound healing of skin, but also induces acute local inflammatory allergic effects when its suspension was injected subcutaneously in rats. This study also reinforced the chemotactic effect of chitin on inflammatory cells suggested before,[224] although it was concluded that it has a positive effect since the onset of acute inflammation wound facilitate early angiogenesis and faster tissue formation and wound healing.[223]

20.6 CONCLUSIONS

There is a demand for the development of new biomaterials that perform their function without eliciting negative effects in the host. One of the major concerns involves the inflammatory response resulting from implantation, which can compromise the success of the implant.

Biodegradable biomaterials have arisen as the solution for some problems which currently involve traditional devices. However, and not neglecting the advantages of the degradable systems, some drawbacks have been found, especially in terms of deleterious effects from their degradation products. Therefore, natural-origin polymers are emerging within the biomaterials field. The rationale is that the incorporation of their degradation products into normal metabolic pathways will avoid secondary effects in the host.

Several strategies, based on some fundamental variables, which are believed to influence the reaction of the tissues, have been used to adjust biodegradable polymers of both synthetic and natural origin, in order to minimize the evoked tissue response. From the materials perspective, the chemistry of the material, the mechanism of chemical and physical degradation, the rate of degradation, reactivity of the leachable degradation products, and the size and shape of the implant have been addressed. Furthermore, other external factors such as animal species and the site of implantation have to be considered.

Although there are many biodegradable biomedical devices being used, it is still unclear which physical or chemical properties of the materials, or which synthetic or natural sources, are responsible for each type of reaction and consequently which are the most appropriate. Thus, it is of utmost importance to consider all variables independently of the synthetic or natural origin of the materials to draw conclusions about the aptness of a potential biomaterial, which has to be adjusted according to the proposed application.

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21 Mediation of the Cytokine Network in the Implantation of Orthopedic Devices

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CONTENTS

21.1 INTRODUCTION

The implantation of a biomaterial into human tissues triggers a set of cellular and biochemical processes collectively known as inflammation, in response to the injury and to the presence of the implant.[1,2] The acute inflammatory response is immediately initiated, leading to exudation of plasma proteins and inflammatory cells that migrate to the site of injury.[3] During this early stage, several chemical mediators control and determine the extent of the reaction which would culminate in the restoration of the tissue and consequently wound healing.[4] However, it is becoming clear that in the presence of an implant the normal healing of injured tissues does not necessarily occur via the same mechanisms.[5] A chronic inflammatory response is often instigated and can be maintained or amplified, depending on the material properties, causing damage of the host tissues.[6–8] This process is mainly controlled by chemical mediators known as cytokines; these substances are produced by the cells present at the implantation site and can act locally or systemically, attracting other cells and inducing the production of other cytokines as well as guiding cellular functions.[9] The so-called cytokine network represents a very complex system of many molecules with multiple actions, involving many different types of cells and intermediaries[10] (Figure 21.1). The same cytokine may

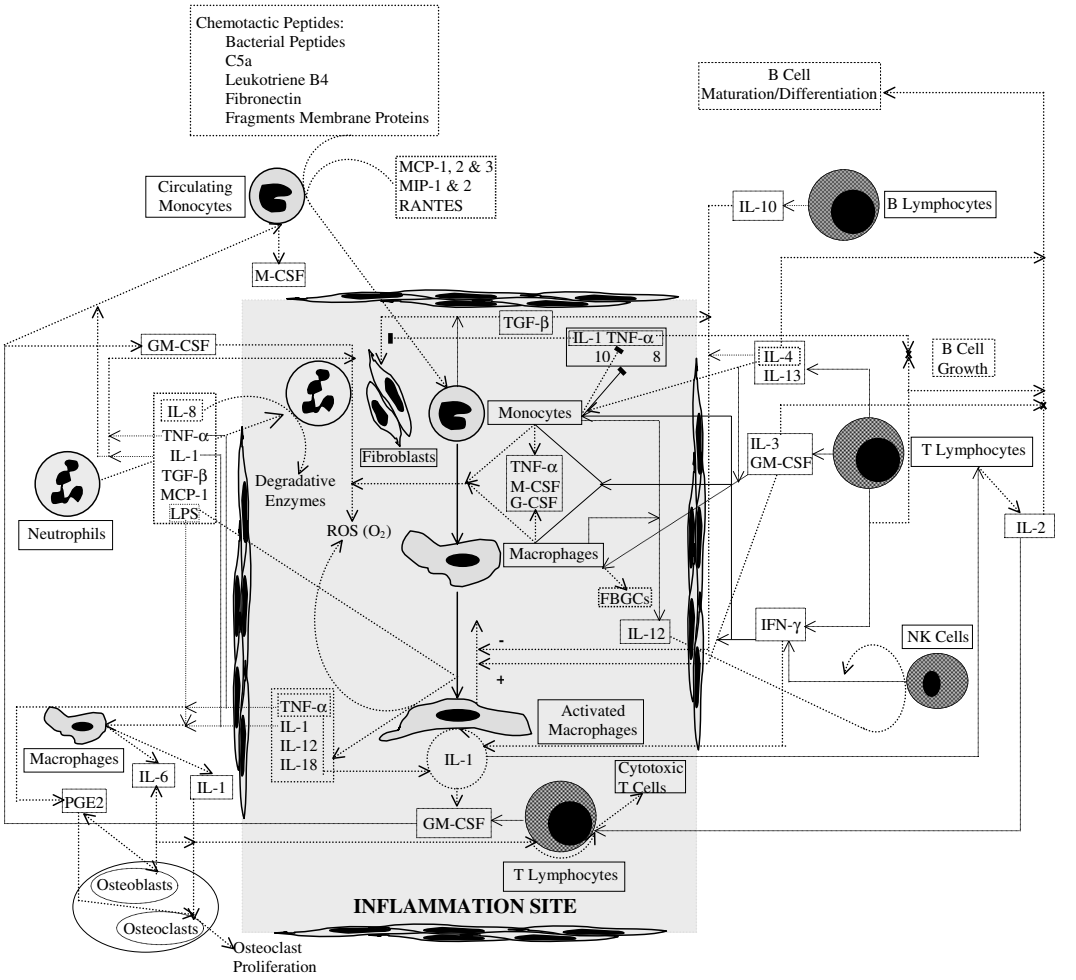

FIGURE 21.1 A schematic representation of some of the biological elements involved in the cytokine network.

act pleiotropically, having different effects on different target cells, while others can act synergistically or antagonistically.[11,12]

Cytokines are commonly catalogued[13] as anti-inflammatory or pro-inflammatory, as immunoregulators that counteract various aspects of inflammation such as cell activation or the production of pro-inflammatory cytokines, or as those that stimulate specific events in inflammation. However, it should be noted herein that this specific classification of cytokines may be misleading. The net effect of an inflammatory response is determined by the balance between pro-inflammatory cytokines and anti-inflammatory cytokines.[13] The type, duration, and also the extent of cellular activities induced by one particular cytokine can be influenced considerably by the nature of the target cells, the environment of a cell, depending, for example, on the growth and activation state of the cells, the type of neighboring cells, cytokine concentrations, the presence of other cytokines, and even on the temporal sequence of several cytokines acting on the same cell.[14–16]

The major drawbacks of current orthopedic implants are localized bone resorption at the bone/implant interface[17,18] and the presence of wear particles,[19] resulting from mechanical abrasion and fatigue. Phagocytes attracted to the area tend to phagocytose particulate wear debris resulting in permanently activated cells producing inflammatory cytokines, which may in turn contribute to the osteolytic process and the loosening of the implant.[20,21]

21.2 CYTOKINES

Cytokines are local protein mediators, involved in almost all important biological processes, namely, cell growth and activation, inflammation, immunity, and differentiation.[14] Molecularly, cytokines are defined as inducible, water-soluble, heterogeneous, proteinaceous mediators, possessing specific effects in target cells or in the mediator-producing cells themselves.[13] Cytokines exert their effects by binding to specific cell-surface receptors which signal to their target cells.[12] They act at very low concentrations (typically 10^{-10} to 10^{-12} *M*), are short-lived, and may act either on other cells (paracrine) or on the same cell (autocrine), or systemically (endocrine).[22]

The term *cytokine* encompasses different classes, interleukins (IL), which refer to a group of cytokines which are typically produced by T lymphocytes and macrophages although other leukocytes are also able to secrete them in lower amounts.[14] Another group of cytokines is designated by the generic name of chemokines due to their effects in the chemotaxis of leukocytes.[22]

However, it is often not clear which molecules should be defined as cytokines, particularly in the case of hormones and growth factors (GF), but the pleiotropic nature of cytokines enabled the problem to be clarified.[14] Furthermore, GF tend to be produced constitutively, whereas cytokine production is carefully regulated and, unlike hormones, which act long range in an endocrine way, most cytokines act over a short distance in an autocrine or paracrine manner.[22]

21.2.1 Pro-Inflammatory Cytokines

The cytokines known collectively as pro-inflammatory cytokines stimulate or accelerate inflammation and also regulate inflammatory reactions either directly or by their ability to induce the synthesis of cellular adhesion molecules or other cytokines in certain cell types.[13] The major pro-inflammatory cytokines that are responsible for early acute phase responses are IL-1α, IL-1β, IL-6, and tumor necrosis factor-α (TNF-α). Other pro-inflammatory mediators include interferon-γ (IFN-γ), transforming growth factor-β (TGF-β), granulocyte and macrophage colony stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), and the interleukins IL-8, IL-11, IL-12, IL-17, IL-18.[13,23]

IL-1 is a major mediator of inflammation and in general initiates or increases a wide variety of nonstructural, function-associated genes characteristically expressed during inflammation.[24] Although secreted by a variety of cells such as activated macrophages from different sources (alveolar macrophages, Kupffer cells, adherent spleen and peritoneal macrophages), peripheral neutrophil granulocytes, endothelial cells, fibroblasts, smooth muscle cells, keratinocytes, Langerhans cells of the skin, osteoclasts, astrocytes, epithelial cells of the thymus and the cornea, T cells, B cells, natural killer cells, monocytes, and tissue macrophages are the main source of IL-1.[22,24,25]

There are two functionally almost equivalent forms of IL-1 — IL-1α and IL-1β — that are encoded by two different genes.[26] IL-1β is the predominant form in humans, while IL-1α is found more abundantly in mice.[13,27] Mature forms of IL-1α and IL-1β and also their precursors are secreted by murine macrophages after stimulation with bacteria or numerous microbial products.[28] Both forms of IL-1 bind to the same receptor and therefore also show similar, if not identical, biological activities.[29] In fact, only a few functional differences between the factors have been described.

Within a few minutes of binding to cells, IL-1 induces several biochemical events. This cytokine is strongly involved in the proliferation mechanisms of several cells acting as a stimulant for NK-cells and fibroblasts and as an inhibitor for endothelial cells. IL-1 causes many alterations of endothelial functions *in vivo*. It promotes thrombotic processes and attenuates anticoagulatory mechanisms. IL-1 therefore plays an important role in pathological processes such as venous thrombosis, arteriosclerosis, vasculitis, and disseminated intravasal coagulation.[30]

Chemotactic properties are also attributed to IL-1; it is a strong chemoattractant for leukocytes, in particular to neutrophils.

TNF-α, another pro-inflammatory cytokine, is produced by activated mononuclear phagocytes, macrophages, and lymphocytes as well as by many other nonimmune cell types.[16] TNF-α is particularly important in organizing reversible microenvironments, and its production can induce

remarkable cellular changes and tissue remodeling.[31] Like IL-1, TNF is a potent activator of neutrophils, mediating adherence, chemotaxis, degranulation, and respiratory burst.[32] However, this cytokine has paradoxical roles in the inflammatory process. While inducing death of diseased cells at the site of inflammation, this cytokine stimulates fibroblast growth.[33,34] In the skeletal system, TNF-α stimulates bone and cartilage resorption and inhibits proteoglycan and collagen synthesis under some conditions.[35]

Together with IL-1, IL-6 is a major physiological mediator of the acute phase reaction inducing hepatic expression of acute phase proteins.[36,37] It is produced by many different cell types, but the most important source is mononuclear phagocytic cells.[38] Macrophages, T and B lymphocytes, granulocytes, smooth muscle cells, eosinophils, chondrocytes, osteoblasts, mast cells, glial cells, and keratinocytes also produce IL-6 after stimulation.[16,22]

IL-6 has pleiotropic functions influencing antigen-specific immune responses and inflammatory reactions. IL-6 is a B cell differentiation factor *in vivo* and *in vitro*, and an activation factor for T cells.[39] However, in contrast to those pro-inflammatory effects, IL-6 also possesses anti-inflammatory functions, namely, inhibition of IL-1, TNF synthesis, and stimulation of IL-1 receptors antagonist (IL-1ra) production.[40]

The most important cytokine responsible for cell-mediated immunity is IFN-γ.[41] The expression of IFN-γ was long considered to be restricted to activated T and NK cells.[42] The production of this cytokine requires activation of the cells, which can occur via a combination of different types of signals; a specific or nonspecific ligand interaction with a T cell receptor, their contact with accessory cells through adhesion molecules, and by a combination of cytokines.[43] Although initially considered to have antiviral functions, it has become clear that IFN-γ has a broader role.[42] Compared with other interferons, IFN-γ growth inhibitory activities are more pronounced, and its main biological activity appears to be immunomodulatory in contrast to the other interferons, which are mainly antiviral.

As with the majority of cytokines, IFN-γ is seen as a pro-inflammatory cytokine with a pleiotropic nature mainly to increase TNF activity and nitric oxide (NO) secretion[44] and to activate the pathways that lead to cytotoxic T cells.[45] IFN-γ exerts important activities on both monocytes/macrophages and lymphocytes, which generally result in macrophage activation and T cell differentiation toward a T_H-1 type of immune response.[45] In addition, it can destroy blood vessels but also induce several angiogenic factors. IFN-γ inhibits the proliferation of endothelial cells and the synthesis of collagens by myofibroblasts, thus functioning as an inhibitor of capillary growth mediated by myofibroblasts, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF).

Besides IFN-γ, another significant lymphocyte-derived interleukin is IL-2, which is produced mainly by T helper (TH) cells, expressing the surface antigen cluster designation 4 (CD4), following activation by mitogen or allogen. Several secondary signals are required for maximal expression of IL-2 and resting cells do not produce IL-2.[16,46] IL-2 induces proliferation of T lymphocytes; however, this only occurs when IL-2 and IL-2 receptors (IL-2r) are simultaneously produced and expressed. Therefore, IL-2 is an antigen-specific proliferation factor for T cells, ensuring that only the T cells specific for the antigen provoking the immune response become proliferative.[16] Due to its effects on T and B cells, IL-2 is a central regulator of immune responses.[47]

21.2.2 Anti-Inflammatory Cytokines

Anti-inflammatory cytokines are generally considered as possessing immunoregulatory and inhibitory properties.[48,49] These mediators act mainly by the inhibition of the production of pro-inflammatory cytokines or by counteracting the many biological effects of pro-inflammatory mediators in different ways.[13] The main anti-inflammatory cytokines include IL-4, IL-10, and IL-13, but other anti-inflammatory mediators include IL-16, IFN-α, transforming growth factor (TGF-β), IL-1ra, granulocyte colony stimulating factor (G-CSF), as well as soluble receptors for TNF or IL-6.[22] Although IL-4, IL-10, and IL-13 are considered anti-inflammatory cytokines due to their ability to suppress production of IL-1, TNF, and chemokines, they are potent activators of B lymphocytes.[13]

IL-4 is produced mainly by a subpopulation of activated T cells (T_H2) which are the biologically most active helper cells for B cells.[22,50] It promotes the proliferation and differentiation of activated B cells,[51] and the expression of low-affinity immunoglobulin E (IgE) receptors in resting B cells.[52] This cytokine can promote their capacity to respond to other B cell stimuli and to present antigens for T cells. This may be one way to promote the clonal expansion of specific B cells and the immune system may thus be able to respond to very low concentrations of antigens.[53]

The anti-inflammatory properties of IL-4 appear to be mediated at multiple levels, directly suppressing the production of pro-inflammatory cytokines,[54,55] as well as antagonizing the pro-inflammatory effects of IFN-γ on several functions.[56,57]

IL-10, secreted by both T_H1 and T_H2 cells,[58] has as major sources in humans monocytes and B cells,[16] and as its main target macrophages. IL-10 suppresses cytokine production by macrophages, thus indirectly reducing cytokine production by T_H1 cells.[16] Furthermore, it down-regulates the expression of major histocompatibility complex (MHC) class II molecules in antigen-presenting cells (APC).[16,59]

IL-13 is homologous to IL-4 and shares a large amount of its biological activities on mononuclear phagocytic cells, endothelial cells, and B cells.[60] This cytokine is, however, more widely produced than IL-4, including by T_H1 lymphocytes, and is readily identified in allergic inflammatory tissue.[61]

21.2.3 Chemokines (Chemotactic Cytokines)

Chemokines are a superfamily of low-molecular-weight proteins that facilitate the passage of leukocytes from the circulation into the tissues.[62] These molecules are capable of inducing chemotaxis in a variety of cells including neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts, and keratinocytes.[16] Despite being considered mainly as chemotactic molecules, their role is much more complex and goes from recruiting and activating leukocytes to homeostatic functions.[16,63] For example, they can have direct effects on T cell differentiation or indirectly by changing APC trafficking or cytokine production.[64]

It was demonstrated that the expression of chemotactic cytokines are both cell and stimulus specific,[65] which suggests that the recruitment of cells to a site of inflammation is dependent upon the expression of specific cytokines for both the induction and maintenance of the lesion.

Four chemokine subfamilies are known[66]: (1) The C-X-C (α) chemokines which includes IL-8 (CXCL8), melanoma growth stimulator (GRO-α), and epithelial neutrophil activating peptide 78 (ENA 78), which primarily target neutrophils; (2) the C-C (β) chemokines such as RANTES (regulated upon activation normal T cell expressed and secreted), MCP-1 (monocyte chemoattractant protein 1), and MIP-1α (macrophage inflammatory protein 1α), which recruit T cells and monocytes; (3) the "C" subfamily, which includes lymphocyte-specific chemotactic peptide XCL1; and (4) the CX3C subfamily with only one member, CX3CL1 (fractalkine).[16]

IL-8 is a potent neutrophil chemoattractant and is the classic chemokine example.[67] It induces adherence to vascular endothelium and extravasation of neutrophils into tissues where they become activated and degranulate, causing tissue damage.[16] In addition, it is a potent inducer of monocyte/macrophage activation, which produces IL-8 in response to IL-1 and TNF.[68]

MIP-1 is a lipopolysaccharide (LPS)-inducible, heparin-binding protein made up of two peptides (MIP-1α and MIP-1β). It possesses chemotactic activity for macrophages[69] and can augment the inflammatory effect of these cells in chronic inflammation.[70]

MCP-1, expressed in macrophages, has similar chemotactic activity and is induced by IL-1 and LPS stimulation of peripheral mononuclear lymphocytes.[71,72]

21.2.4 Cytokine Growth Factors

Cytokine GF are produced by a variety of cells, including those typically involved in inflammatory processes, such as macrophages,[73] lymphocytes,[74] endothelial cells,[73] platelets,[73,75] and fibro-

blasts.[76,77] These cytokines are powerful activators of the production of collagen and other extracellular matrix components, often in an autocrine manner.[77]

Colony stimulating factors (CSF) are examples of cytokines with direct effects on cell proliferation, in particular the stimulation of growth of colonies of cells from bone marrow precursors.[78] CSF-1, also referred to as macrophage colony stimulating factor (M-CSF), promotes the growth of macrophages, while GM-CSF promotes the growth of both granulocytes and macrophages.[78,79] CSF-1 appears to be an important signal in inducing monocytes to mature into macrophages.[80]

TGF-β is a member of one of the most complex groups of cytokine superfamilies, consisting of various TGF-β isoforms and other family members, for example, Activin A and bone morphogenic proteins (BMP).[81] This family of cytokines is produced primarily by chondrocytes, osteocytes, fibroblasts, platelets, monocytes, and some T cells.[16] It has both stimulatory and inhibitory effects on different cell types.[82]

21.3 ADHESION MOLECULES

Cell adhesion molecules (CAMs) play an essential role in adhering circulating leukocytes to the vascular endothelium at the sites of inflammation and their subsequent transmigration into adjacent tissues. In the absence of signals to stimulate the expression of CAMs, the adhesive forces between endothelium and leukocytes are not enough to attach leukocytes.[83]

The adhesion molecules can be divided into three families of different structural architecture: selectins, integrins, and certain glycoproteins included in the Ig superfamily.[84]

Cytokines have been implicated in the up-regulation of many CAM's expression, increasing the adhesiveness between leukocytes and endothelium, which may be crucial to the regulation of inflammatory processes.[85] Cytokine-activated endothelial cells also secrete chemokines such as IL-8 and MCP-1 required for leukocyte recruitment.[86]

21.3.1 SELECTINS

Selectins are molecules that mediate attachment of leukocytes and platelets to vascular surfaces. They are characterized by an extracellular motif involving two domains: a lectin-like domain attached to an epidermal growth factor (EGF)-like domain and a variable number of complement regulatory protein repeated sequences.[84,87–89]

There are three selectins which have been shown[90] to be important in the cell-to-cell adhesion process; L-selectins are constitutively expressed on leukocytes, while E-selectins are present exclusively in endothelial cells, being only expressed following stimulation by cytokines.[90] In contrast, P-selectins are accumulated preformed for rapid release in platelets or endothelial cells.[91]

Selectins play a critical role in the leukocytes initial attachment and rolling on the vascular endothelium prior to integrin action.[92] Endothelium becomes activated by inflammation-induced cytokines, in turn resulting in the expression of selectins.[84] The interaction of P- and E-selectins with the carbohydrate ligands on the surface of leukocytes appears to be responsible for initiating their rolling on the endothelium.[84] Although neutrophils and some lymphocytes constitutively express L-selectin, it is only after E- and P-selectin expression by endothelial cells that the rolling process occurs.[89,92]

21.3.2 INTEGRINS

Integrins are heterodimers consisting of noncovalently linked α and β subunits.[93] There are many possible combinations between the different known subunits; however, β_2, $\alpha_4\beta_1$, and $\alpha_4\beta_7$ integrins are the main intervenients in regulating immune cell adhesion to endothelium.[84]

Within the β_2 integrins, it is possible to find the surface antigen expressed in all leukocytes, known as lymphocyte function-related antigen (LFA-1); it is an integrin consisting of a α subunit

(designated CD11a), and the β_2 subunit (designated CD18).[83] The α subunit can vary in the heterodimer region, originating two other important adhesion molecules, the Mac-1 (designated CD11b) and C3b receptor (designated CD11c), both of which are expressed on monocytes/macrophages and granulocytes[94] but not on lymphocytes.[95] CD11a is involved in the adhesion of leukocytes to endothelium, and Mac-1 plays a key role in the adherence of both monocytes and neutrophils to vascular endothelium for subsequent extravasation.[96,97] CD11b/CD18 is also implicated in a variety of cell–cell and cell–substrate interactions such as attachment and phagocytosis of particles coated with C3bi by granulocytes and macrophages.[98]

The α_4 subunit-containing integrins have been termed VLA (very late activation) since two of them are expressed on lymphocytes about 2 weeks after antigen stimulation *in vitro*.[98] VLA-4 ($\alpha_4\beta_1$; CD49d:CD29) is expressed in resting lymphocytes and monocytes and is probably the most important VLA integrin with respect to cell adhesion.[83]

The $\alpha_4\beta_7$ integrin, also known as lymphocyte Peyer's patch adhesion molecule-1 (LPAM-1), is expressed on the microvillus tips of lymphocytes mediating the adhesion to the walls of inflamed venules.[84]

It is important to emphasize that the adhesion and consequently the cell migration to a site of injury via integrins is dependent on the changes which occur in the adhesion molecules subunits. Rather than an increase in the amount of expressed CAMs, stimuli-like cytokines and antigens induce a conformational change of the integrins from a low- to a high-affinity state.[84]

In addition to their role in the adhesion of cells to endothelium, integrin receptors on the neutrophil cell surface facilitate binding of neutrophils to the extracellular matrix.[99]

21.3.3 Immunoglobulin Superfamily Membrane Proteins

Membrane proteins belonging to the Ig superfamily are specific cell surface molecules which act as counter-ligands for integrins. Those which are expressed on endothelial cells can be also designated as Ig-like addressins.[83]

Some representative examples of membrane protein members of the Ig superfamily are[22] Ig-α/Ig-β heterodimer, part of the B cell receptor, T cell receptor (TCR), T cell accessory proteins such as CD2, CD4, CD8, CD28, and the γ, δ, and ε chains of CD3, class I and class II MHC molecules, PDGF and various cell-adhesion molecules, including vascular cell-adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), ICAM-2, and LFA-3.

21.4 CYTOKINES REGULATION

The same cytokine may act pleiotropically, having different effects on different target cells or sometimes even on the same cell, while others can act synergistically or antagonistically for the same result[23] (Figure 21.1). The effect of a particular cytokine depends on the context in which it is working, since it is unlikely that cells in a particular inflammatory situation are exposed to only a single cytokine or only one inflammatory mediator or even one cell type.[100]

The first cells to appear in a site of inflammation are neutrophils. In fact, neutrophil numbers reach peak levels approximately 24 hours after injury.[2] Their migration is stimulated by various chemotactic factors and cytokines, including complement factors, IL-1, TNF-α, TGF-β, and chemokines such as IL-8 and MCP-1, and also by bacterial LPS.[101,102] IL-8 and IL-1, besides being chemoattractants for neutrophils, respectively induce degranulation and activate the oxidative metabolism of those cells, causing tissue damage.[16] In addition, pro-inflammatory cytokines such as GM-CSF and TNF-α modulate NADPH oxidase activity, inducing the release of large quantities of superoxide anion (O_2^-)[103] in a phenomenon known as the respiratory burst. Regulation of this free-radical production is critical to kill pathogens without inducing tissue injury.[103]

Monocytes enter inflammatory sites where they develop into the macrophage under the influence of a number of inflammatory mediators derived from other migrating cells (lymphocytes,

macrophages, and granulocytes) as well as from the affected tissue and endothelial cells themselves.[11] These are the cells that essentially control and regulate the wound-healing process and wounds cannot heal without the participation of these cells, as shown by experiments involving depletion of wound macrophages.[104,105]

Monocytes are recruited following interaction with chemotactic peptides such as bacterial peptides, complement fragment C5a, leukotriene B4, fibronectin, and fragments of basement membrane proteins.[106] Chemokines such as MCP-1, -2, and -3, MIP-1 and -2, and RANTES also contribute to the recruitment of circulating monocytes within tissues.[11] The profile of cytokines secreted by activated and resident tissue macrophages is different,[107] which allows the modulation of most of the macrophage functions and cell surface marker expressions. Some cytokines (IL-3, GM-CSF, IFN-γ) can up-regulate the production of other cytokines by macrophages, while IL-4, IL-10, IL-13, and TGF-β can inhibit that secretion.[13] TNF-α, IFN-α, IFN-β, and IFN-γ and also bacterial endotoxins, viruses, mitogens, and antigens induce the synthesis of IL-1.[16,28] In human monocytes, bacterial LPS induce approximately tenfold more mRNA and the respective proteins for IL-1β than for IL-1α.[27] IL-1 can also induce the synthesis of GM-CSF by peripheral blood lymphocytes and synergizes with that cytokine in the induction of M-CSF.[108] In infection, besides IL-1, TNF-α, IL-12, and IL-18, production is also stimulated by LPS. IL-12 is generally considered the major inducer of IFN-γ production by T and NK cells.[42,109] Pro-inflammatory IFN-γ stimulates the bactericidal activity of phagocytic cells and therefore boosts the innate response.[41] In monocytes and macrophages, besides the secretion of TNF-α, IFN-β, IL-1α, and β,[110–112] IFN-γ induces the transcription of genes encoding G-CSF and M-CSF and also stimulates the release of reactive oxygen species (ROS).[44] In addition to those stimulatory effects, IFN-γ can exert some inhibitory activity on the production of other inflammatory mediators such as IL-1, IL-2, IL-8, IL-10, and MCP-1, in human monocytes.[15,113–115] Bacterial endotoxins together with IL-1, TNF, PDGF, and Oncostatin M also represent physiological stimuli for the synthesis of IL-6.[116] The synthesis of IL-6 in human alveolar macrophages is, however, inhibited by IL-4, which prevents the production of IL-1, TNF-α, and prostaglandins in response to activation of the cells by bacterial endotoxins or IFN-γ.[117] Furthermore, IL-4 induces the formation of foreign-body giant cells (FBGC) from human monocyte-derived macrophages *in vitro,* which can in turn be reinforced by the action of GM-CSF and IL-3.[118]

The expression of certain cell surface markers has been often addressed[119,120] as an index of cellular immune function and suppression and shown to be influenced by cytokine action.[121–127] Inflammatory mediators such as TNF-α,[123] IL-1α,[126] IL-4,[124] IL-10,[125] and prostaglandins[121] are known to regulate MHC-II expression. IFN-γ also regulates the expression of MHC class II genes and is the only interferon that stimulates the expression of these proteins. Due to a direct correlation with depressed MHC-II expression and defective antigen presentation,[123] a monocyte population with up-regulated MHC-II expression is important for certain healing processes. IL-4 down-regulates the expression of CD14 in normal human monocytes but strongly increases the expression of CD23, another monocytic antigen.[122]

IL-1 and TNF-α are also responsible for increasing the expression of adhesion molecules,[127] which allow leukocytes to adhere to endothelium prior to their extravasation into tissues. IL-1 promotes the adhesion of neutrophils, monocytes, T cells, and B cells by enhancing the expression of ICAM-1 and endothelial leukocyte adhesion molecule (ELAM).[16] Lymphocytes use LFA-1 and VLA-4 to respectively bind ICAM-1 and VCAM-1.[128] In turn, neutrophils appear to use both LFA-1 and Mac-1 to attach to ICAM-1 expressing cells.[84] ICAM-1 is primarily recognized by β2 integrins while the VLA-4 molecules interact with VCAM-1.[84] Finally, the LPAM-1 integrins recognize the mucosal addressin cell molecule-1 (MAdCAM-1).[84] In addition, the transmigration through the intercellular junction of endothelial cells appears to require the expression of platelet/endothelial cell-adhesion molecule-1 (PECAM-1), another molecule belonging to the Ig superfamily.[129]

Lymphocytes are together with neutrophils, monocytes, and macrophages involved in the inflammatory process.[130] Appropriate T_H cell development is essential for an effective adaptive

immune response. It is now established that soon after microbial invasion, macrophages promptly secrete considerable amounts of IL-12, which triggers the differentiation of T cells toward a T_H1-type response.[131] Although the understanding of immune regulation is incomplete, it is known that CD4+ helper T cells are capable of differentiating from an initial common state (T_H0) into two apparently distinct types called T_H1 and T_H2, which differ in their cytokine secretion.[16,132,133] T_H0 are responsible for the secretion of IFN-γ, IL-2, IL-3, IL-4, GM-CSF, IL-5, IL-10, and TGF-β. After differentiation, IL-2, IL-3, and GM-CSF continue to be produced by both subsets, while IFN-γ is secreted only by T_H1 and IL-4, IL-5, and IL-10 and TGF-β by T_H2. In addition, two other cytokines, LT-β and IL-6, are produced respectively by T_H1 and T_H2 cells.

The balance between T_H1 and T_H2 represents a switch, which can be used to influence the immune response in one or other direction. The commitment of T_H0 cells to become T_H1 or T_H2 is influenced by cytokines secreted by the 2 subtypes themselves and by macrophages, NK cells, and mast cells.[134]

The T_H1 pathway is essentially cell-mediated immunity, with the activation of macrophages, NK cells, cytotoxic T cells, and a prolonged inflammatory response. A main biological activity of IL-1 is the stimulation of T_H cells, which are induced to secrete IL-2 and to express IL-2 receptors.[16] In the presence of IL-2, IL-6 induces the differentiation of mature and immature T cells into cytotoxic T cells.[135] The expression of the IL-2 receptor of monocytes is modulated by IL-5 and IL-6 and induced by IFN-γ, so that these cells become tumor-cytotoxic.[136] IFN-γ thus influences cell-mediated mechanisms of cytotoxicity modulating T cell growth and functional differentiation. It is a growth-promoting factor for T lymphocytes and improves the response of these cells to mitogens or GF. In addition, IFN-γ acts synergistically with IL-1 and IL-2[137] and appears to be required for the expression of IL-2 receptors on the cell surface of T lymphocytes.[138]

The T_H2 pathway is essentially a humoral pathway, with the production of cytokines, which promote B cell growth (like IL-4, IL-6) and the production of IgG1 (IL-4), IgA (IL-5), and IgE (IL-4) in mice.[139] It also stimulates effectors, which use these antibody isotypes, eosinophils (via IL-5), and mast cells (IL-4). IL-4 plays a pivotal role within this pathway. In activated B cells, IL-4 stimulates the synthesis of IgG1 and IgE and inhibits the synthesis of IgM.[140] This isotype switching induced by IL-4 in B cells is antagonized by IFN-γ.[16] IL-2 promotes the proliferation of activated B cells, but this requires the presence of additional factors, for example, IL-10.[141] IL-6 is capable of inducing the final maturation of B cells into immunoglobulin-secreting plasma cells if the cells have been preactivated by IL-4. The growth of B cells induced by IL-4 is, however, directly inhibited by the synergistic action of IFN-γ, TNF-α, and TNF-β.[142] IL-3 also inhibits the proliferation of human B cells stimulated by IL-2 by antagonizing the IL-2-induced effects in B cells and by causing a slow decrease of the expression of IL-2 receptors.[143] In contrast, IFN-γ and Anti-Ig costimulate the proliferation of human B cells although not of murine B cells.

Macrophages also control the degradation of the extracellular matrix and regulate remodeling of the wound matrix by secreting several neutral proteinases such as elastase, collagenase, and plasminogen activators.[144] Fragments of extracellular matrix and fibrin degradation products can be phagocytosed and degraded, which together with the cleavage of plasminogen into plasmin, results in the onset of important inflammatory processes involving not only activation of fibrinolysis, but also the complement, kinin, and coagulation cascades.[145] Macrophages also secrete GF such as TGF-β and PDGF, which have been shown to stimulate the growth of fibroblasts. TGF-β appears to be the major factor responsible for the formation of granulation tissue and the synthesis of proteins of the extracellular matrix,[16] which have lead to it being attributed as a wound hormone. TGF-β1 and TGF-β2 inhibit IL-1-mediated proliferation of lymphocytes and at the same time decrease the secretion of inflammatory proteins such as neutral proteases. Therefore, IL-1 activity antagonizes the effects of TGF-β on the extracellular matrix.[146] The complexity of the wound-healing process is illustrated by the observation that manipulation of the ratios of TGF-β superfamily members, particularly the ratio of TGF-β-1 relative to TGF-β-3, reduces scarring and fibrosis.[147] Reepithelialization is mediated by chemotactic and mitogenic GF of the EGF family of GF.

The mechanism of inflammation seems rather straightforward, but how the immune system regulates the type of response to a given challenge is still unclear. Different types of challenges require very different protective mechanisms to be activated; an inadequate response can fail to protect the host against an organism or even cause damage to the host directly. Systemic actions can be critical and not only cells and chemical modulators at the site of inflammation are involved in the process. Cell such as monocytes/macrophages and endothelial cells also contribute to bone remodeling by either contact with osteogenic cells or by the release of soluble factors, namely, cytokines and GF.[148]

In the skeletal system, TNF-α stimulates bone and cartilage resorption and inhibits proteoglycan and collagen synthesis under some conditions.[35] IL-1 induces the expression of a large variety of cytokines. LIF and IL-6 are two of those molecules which are known to stimulate mesenchymal progenitor differentiation toward the osteoblastic lineage,[149] but they are also potent antiapoptotic agents of osteoblasts.[150] In bone, the major sources of IL-6 are osteoblastic cells and not osteoclasts[151]; however, the main activity of that cytokine involves osteoclastogenesis and bone resorption.[152] PGE2 is also directly related to IL-6 expression.[153]

The production of IL-1, TNF-α, and TGF-α is influenced by prostaglandins, in particular its ability to stimulate bone resorption which is mediated by increased prostaglandin E_2 (PGE2) synthesis.[154] Large amounts of PGE2 are produced in cells stimulated with IL-1.[155] In fact, many of the biological activities of IL-1 are due to an increase of PGE2 production.[13] *In vitro*, PGE2 and glucocorticoids inhibit the synthesis of IL-1. The PGE2-mediated inhibition of IL-1 synthesis, like the inhibition of IL-1 synthesis caused by IFN-α and IFN-γ, is mediated by an increase of intracellular cAMP levels.

The effects of TNF-α, IL-1, IL-6, and PGE2 are therefore interconnected; IL-6 stimulates osteoclast formation inducing the release of IL-1[156] and mediates the stimulatory effects of TNF.[157] PGE2 together with IL-6 activates osteoclasts in a paracrine way. These cytokines act synergistically in the stimulation of osteoclast differentiation acting on the stromal cells or directly on osteoclasts and their precursors.

21.5 ORTHOPEDIC APPLICATIONS

The challenge in the development of new devices for orthopedics is to ensure long-term stability, anchorage, and function. Loosening of joint prosthesis resulting in failure is a major concern in the biomaterials field for orthopedic applications[158,159] with revision surgery occurring at early or later stages of implantation, depending on the cause of failure. Key factors are believed to be the generation of wear particles and the biological response to them in periprosthetic tissues,[160] as well as the degradation products of biodegradable materials which result in osteolytic reactions.[161] These reactions modulate the formation and resorption of mesenchymal tissue and eventually lead to some of the pathological findings in failed total joint replacements including membrane formation, periprosthetic osteolysis, and implant loosening.[17,162,163]

The adverse effect of currently used orthopedic devices was suggested to depend more on the particulate/degradation products[164,165] nature of the material than its chemical biocompatibility.[166] For example, both the size and volume (or number) of polyethylene particles are critical factors in macrophage activation and particles in the phagocytosable size range of 0.3–10 μm appear to be the most biologically active.[167] A similar result was found for a given mass of polymethylmethacrylate (PMMA) bone cement; smaller particles (less than 20 μm) resulted in more inflammation than larger particles (50–350 μm),[168] and irregularly shaped particles produced a greater response than spherical particles. Furthermore, large particles induced a more intense increase in the white blood-cell count and in the production of PGE2.[168] Other work[169] indicates that most of the particles in implant membranes are smaller than the resolution of the light microscope and that tissue digestion is necessary for quantification. The concentration of polyethylene particles accumulated in the tissue has been concluded by some research to be the most critical factor in the pathogenesis of osteolysis.[170]

Thus, there is not yet any general understanding of the mechanisms by which particulate materials exert a harmful effect greater than that of the whole material. Hydroxyapatite products, well tolerated in bulk form, have been used widely in clinical medicine. However, porous HA blocks have an unacceptably high failure rate in clinical applications.[171] TGF-β1 concentration was found to decrease by the addition of HA particles *in vitro,* but that variance was dependent on particle size.[172] In addition, that effect was also suggested to be mediated by the increased synthesis of PGE2.[154,172]

There have been many studies on tissue at the bone/cement–material interface,[163,173–175] and attention has been focused on analyzing the retrieved specimen to measure the material degradation from the real environment and to perform biological studies on the tissues. In some of the studies, the tissue was found to be fibrous granulation tissue[173,175,176] with degradation particles being released into surrounding tissue. These particles can initiate chronic inflammation with a significant number of activated macrophages and FBGC aiming to eliminate the debris.[165,177] Therefore, implant-derived wear and degradation particles are thought to induce cytokines and prostaglandins which are the primary cause of osteolysis.[178–181] In fact, retrospective studies on failed implants suggest that periprosthetic osteolysis is mediated by activated macrophages and consequently by the released cytokines.[182] Other works[20,175] have revealed that several cytokines are produced in that tissue, which suggests a critical role for cytokines in bone destruction and total hip arthroplasty (THA) loosening.

Demonstration of the production of bone resorptive cytokines in response to wear debris does not, however, demonstrate that bone resorption is only increased by this mechanism, since wear particles also induce production of factors that inhibit bone resorption such as IL-4 and IL-10.[183] IFN-γ, for example, is involved in the processes of bone growth and inhibits bone resorption probably by partial inhibition of the formation of osteoclasts. At the same time, IFN-γ synergizes with LPS in the induction of NO production.

Particulate wear debris and degradation products have been shown to alter the function of a variety of cell types within the periprosthetic space including macrophages, fibroblasts and osteoblasts, and either directly or indirectly osteoclasts.[21,181,184,185] Particulate debris induces monocyte/macrophage activation by multiple signaling pathways.[186,187] The interaction between particulates and cell membrane increases cytokine release without requiring phagocytosis.[186] In addition, the selective opsonization of orthopedic implant wear particles by human serum proteins was also shown to influence monocyte/macrophage activation.[187]

Strong evidence has been shown[185,188,189] for the major role of increased recruitment of osteoclast precursors, namely, macrophages, and their subsequent role in implant-induced osteolysis, while osteoclast activation and survival appear to play minor roles. In addition, a synergistic effect of cell activation and wear particles on O_2^- production by activated macrophages and osteoclasts, suggested O_2^- involvement in mediating osteolysis.[190] Osteoclasts are capable of producing ROS, which were suggested to play a role in normal bone resorption at the osteoclast–bone interface.[191] Low levels of ROS play a role in the differentiation of preosteoclasts and thus, if produced by macrophages or osteoclasts in response to cytokines and wear debris, can increase osteoclast formation.[190,192] Furthermore, EGF and TGF-α[193] mainly responsible for wound healing, can induce bone resorption partly due to their ability to increase the proliferation and fusion of osteoclast precursors, leading to an increase in the number of osteoclasts.[194,195]

Nitric oxide seems to play a role in stimulating resorption of bone by macrophages and osteoclasts.[196–199] Analysis of revision tissue has identified the presence of functional inducible NO synthase in activated macrophages and endothelial cells containing metal, polyethylene, and polymethylmethacrylate (bone-cement) particles.[200,201] Nonetheless, the effect of biomaterials on macrophage production of ROS and reactive nitrogen species is largely unexplored. Those species are known to damage extracellular matrix and to increase their degradation by proteases,[202,203] but they also elicit an increase in cytokine production at the implant–bone interface.[204] During inflammation, ROS have been reported[205] to activate collagenase and initiate bone resorption, another finding to support their role in osteolytic processes that cause aseptic loosening.

Macrophages respond to wear particles by releasing the pro-inflammatory cytokines IL-1, IL-6, and TNF-α, as well as other bone-resorptive fibroblast-derived mediators such as PGE2 and matrix metalloproteinases.[18,206–208] Cemented prostheses showed higher incidence of severe osteolysis, and higher level of cytokines.[209] It has also been reported[210] that bone resorption occurred as a result of the macrophage inflammatory response to particulate polyethylene. Local bone-resorbing agents such as IL-1α,[211,212] IL-1β,[211,213,214] TNF-α,[211–213] and LPS[211,212,214] greatly induced IL-6 mRNA expression in both cell line and primary osteoblast cells.

In combination with TNF-α, IL-1 appears to be involved in the generation of lytic bone lesions.[215] IL-1 activates osteoclasts and therefore suppresses the formation of new bone.

Osteoblasts are exposed to the cytokines released in the periprosthetic space. TNF-α[216,217] and PGE2[216,218] have demonstrated negative impact on a variety of osteoblast functions such as suppressing procollagen α1 mRNA expression[216–218] and subsequent reduction of type I collagen synthesis.[218] TNF-α induces the release of IL-6,[219] IL-8, MCP-1, and TGF-β. Secreted IL-6, together with PGE2, activates osteoclasts. In addition, a direct effect of wear debris on osteoblasts showed increased production of IL-6 and PGE2 and a direct influence in bone collagen mRNA expression and on the biosynthesis of bone collagen.

Lukacs et al.[220] reported MCP-1 and MIP-1 production by fibroblasts in inflammatory granuloma and these chemokines were also found in membranes retrieved from total joint arthroplasty.[221] Considering that fibroblasts are a source of C-C chemokines, they can act as chemoattractants for inflammatory cells in response to wear debris. Fibroblasts may also play an important role in osteolysis by increasing the synthesis of metalloproteinases and the secretion of certain mediators that suppress the expression of collagen.

In some cases of aseptic loosening, T lymphocytes were shown to be present, together with debris-containing macrophages, which suggests a sensitivity reaction to those particulates.[199] The recruitment of T lymphocytes to tissue interface membranes of aseptic loosened devices was confirmed by another work although those cells were not participating in hypersensitivity responses.[18] Furthermore, it was found that, in mice,[222] there is a lymphocyte-independent pathway of macrophage activation in response to particulate polymethylmethacrylate. This suggests that the foreign-body response to particulate orthopedic biomaterials is macrophage-dependent and that lymphocytes are not essential to this response, although they may modulate it.

Bone resorption results in further loosening of the prosthesis, changes in stress, frictional wear, release of more wear debris, and recruitment of more macrophages.[223] Bone death and proliferation of macrophages thus appear to be the cause for pain and loosening of prosthesis.

Because many promising materials and designs have failed in clinical use, an understanding of the mechanisms involved in osteolysis is crucial to the development of new methods to prevent implant loosening. Extensive theoretical and experimental testing is mandatory before introducing new materials and implants in a clinical application. To date, many different materials have been tried in order to reduce wear and the generation of macrophage stimulating submicron-sized particles, or to provide more biocompatible components. Therefore, several studies have been carried out with potential biomaterials in order to try to understand which conditions can modulate inflammatory cell activity in response to the implanted material. By testing the cells that give rise to particular tissues rather than the tissue itself, the biological effects of biomaterials on the soft tissue can be elucidated.

Immediately following implantation, proteins adsorb onto the surface of the device; therefore, the effect of the type and amount of proteins as well as the dynamics of adsorption on cytokine production has been in focus in several published works.[224,225] The hypothesis of controlling the inflammatory response of implanted devices has emerged. Natural and synthetic polymers, with variable and selective protein adsorption, have been used to coat other materials expecting to "passivate" within certain limits those materials.[226] The surface chemistry of a biomaterial implant can determine the degree of monocyte and macrophage adhesion and consequently the types and levels of secreted cytokines.[225,226] Therefore, the surface chemistry of the material directly or indi-

rectly dictates monocyte adhesion and macrophage activation and fusion by determining the type, amount, and conformation of adsorbed proteins. Brodbeck et al.[227,228] showed that hydrophilic and anionic surfaces promote an anti-inflammatory type of response *in vitro*[227] and decreased rates of monocyte/macrophage adhesion and fusion *in vivo,*[228] proving that biomaterial-adherent cells undergo biomaterial-dependent responses, sometimes affecting the surrounding implant environment.

However, there are still some reservations in drawing conclusions, not only because the *in vitro* studies can be considered limited due to the lack of independent and dependent variables or to simplistic conditions which do not represent the *in vivo* environment, but also because some results may seem contradictory.[23,24] For example, polymers that supported the highest number of adherent monocytes also elicited the lowest levels of pro-inflammatory cytokines secretion.[226] Conversely, chitosan-based hydrogels were found to inhibit the adhesion of macrophages, maintaining their viability without significantly affecting the production of IL-6 and TNF-α.[229] However, cytokines *in vivo* rarely, if ever, act alone.

Another concern involves the secretion profile of macrophages, which is dependent on their stage of differentiation and on environmental stimuli.[107] Human macrophages obtained from various anatomical sites in the absence or presence of an inflammatory reaction also show differences in their spontaneous and stimulated release patterns.[230,231] Differentiation of monocytes *in vitro* in the presence of various stimuli such as bacterial antigens, lymphokines, and monokines alter their subsequent secretory pattern upon stimulation with membrane-activating agents.[232]

21.6 CONCLUSIONS

Revision surgery of orthopedic implants is becoming a rather typical occurrence. There are multiple factors such as mechanical, biomechanical, and host-specific biological factors, which can be responsible for implant failure. Early failure may occur due to infection, surgical technique, or mechanical overload. However, aseptic loosening that appears several years after implantation seems to involve different mechanisms. The hypothesis involves inflammatory reactions to the implant, in particular to the wear debris particles, which lead to osteolysis, a critical process in implant loosening.

Many uncertainties still exist about the role and the relationship of each part involved in the process of aseptic loosening of an orthopedic implant. Awareness of a pro-inflammatory cascade, which might be regulated by pro-inflammatory factors together with other anti-inflammatory factors, has increased. However, the sequence of events and the parameters influencing the interactive process are still without the means to control them.

Many different materials with demonstrated potential *in vitro* have been proposed, but it is very important to understand that *in vitro* conditions can be very limited and therefore may not be representative of the *in vivo* situation. Therefore, it might be valuable to identify *in vitro*, the most important parameters involved in the circumstances studied, in particular with a specific material, and to use *in vivo* systems to understand their interactions and the net effect.

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22 Protein and Cell Interactions with Biodegradable Systems

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CONTENTS

22.1 SURFACES, SOLUTIONS, PROTEINS, AND CELLS: THE FOUR KEY ELEMENTS

Whenever a protein solution comes in contact with a solid surface, molecules spontaneously accumulate at the solid–liquid interface. In recent decades, protein adsorption has been reported by several authors[1–5] as the initial step following the contact of an artificial surface with blood. This phenomenon was quickly related to the initiation of thrombosis by foreign surfaces,[6] and its dynamics and complexity were initially pointed out in the observations of Watson and Sodersquist[7] as by the Vroman effect concept.[8] Protein adsorption was earlier found to alter the sorbent surface and in many cases also the properties of the adsorbed molecules.[9] More specifically, the adsorption of a certain protein to a surface is often accompanied by a change in its structure or three-dimensional rearrangements. The protein–surface interaction has been of major concern in a number of fields such as medicine,[10–12] pharmacology,[13,14] and biotechnology,[15–17] as several biological processes depend on protein adsorption onto biosurfaces.

During recent years of research in the biomaterials field, predicting, controlling, and manipulating protein adsorption onto biomaterial surfaces has been one of the main aspirations. New experimental techniques were developed and the design of theoretical and descriptive models could in some cases be achieved.

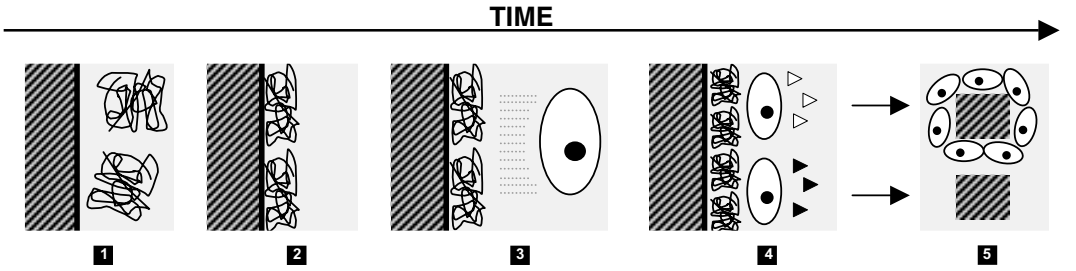

FIGURE 22.1 Schematic representation of the interaction between surfaces, proteins, and cells. Legend: proteins and surface before (1) and after (2) interacting; the proximal cells (3), by means of interacting with the surface/protein layer, initiate signaling mechanisms (4), which can lead in the end to a cell covering or to a cell-resistant surface (5).

The definition of what is desirable or undesirable regarding protein adsorption is evidently related to the application one is dealing with. In turn, this same application success is intimately defined by the biological reactivity starting from the surrounding environment: the fluid composition and the cellular profile.

In the biomaterials field, the performance of several devices depends on different aspects of the protein adsorption phenomena that affect cell response and determine the implant performance (see Figure 22.1). On the other hand, controlling the adsorption of proteins from aqueous solutions or from the blood serum is only viable by means of a complete understanding of its specific properties, organization, and dynamic protein-interface mechanisms. In turn, this demands a detailed characterization of the biomaterials surface composition and molecular structure, since the primary interactions between a biomaterial surface and the biological environment occur in the atomic level and in a very thin interface of less than 1 nm in thickness.[18]

Early on, under this perspective, the opinion of the authors was that there was already the need for plenty of information to complete a protein adsorption study. Empirical answers were to be achieved[19]: What is the classical protein adsorption isotherm? What is the adsorbed amount as function of time? What is the protein orientation 3-D conformation? How do proteins compete with one another? Do proteins desorb or exchange? How does the surface change all this? How do surface–protein systems modulate cell response?

In this review chapter, a comprehensive overview on the process of protein adsorption is presented, from one-protein systems to the complex multi-protein environment, tracing protein dynamics, instability, practical details, limitations, and the success of adsorption manipulation on the control of cell response. Finally, the surface–protein interface is considered and presented in terms of its relevance to understand the biological performance of biodegradable materials. Furthermore, complexity, concerns, and potential problematic issues are also described.

22.2 THE IMPORTANCE OF SURFACE PROPERTIES

Polymers are macromolecules composed of many small monomers added to each, forming linear, branched, or crosslinked structures.[20] Many of the most popular natural-origin polymers are polysaccharide-, protein-, or polynucleotide-based structures.

These are manly distinguished by their degradation rates. In the biomedical field, the control and rate of degradation is critical for the assigned function. Chapter 1 reviews biodegradable materials applications and properties; and further information on natural-origin biomaterials can be found in Chapters 11, 19, and 20.

The human body is a hostile environment for implanted polymers, due to the reactivity that fluids and biological surfaces develop.[19,21] The surface and, to a later extent, the bulk of the material will undergo significant changes starting from time zero of implantation.[19,22–24] Biodegradable

materials are susceptible to incorporating ions and compounds from the surrounding environment, but also to send to solution products of the degradation.[23] Material surface can easily alter the phenotypic expression of bone-related cells; namely, molecular weight, polydispersity, wettability, and crystallinity can perturb normal cell bioprocesses.[25,26] On the other hand, local tissue response initiate surface erosion mechanisms originating degradation by-products, which can affect the pH of the neighboring surroundings.[27] The entire environment that is settled around the implanted device will affect the overall cell response conditioning tissue activity and, finally, the recovery of the patients health condition.[28,29]

One of the most well-known examples of applications of biodegradable systems in clinical situations, is on the field of bone-related substitution.[30–32] Bone is known to phase between formation and resorption, a turnover process of osteoblast-osteoclast interactive cycles.[33,34] This balanced synchronism involving these two mechanisms is controlled and extremely important for bone normal development. Several pathologic condition bioprocesses arise simply from disturbing normal bone homeostasis.[35–37] When biodegradable biomaterials are implanted, its rate of resorption or degradation must go together with bone formation and moreover, the decrease in the mechanical properties of the device are required to protect the bone tissue by simultaneously reducing its strength.[38] In other words, a balance between rigidity, strength, and elasticity of bone and material properties is to be scaled.[39–41] Further information on bone biology can be found in Sections 2.1 and 2.2 of Chapter 2.

Protein adsorption will obviously have a profound effect on the biostability and interfacial properties of the implanted surface, including surface tension, water affinity, or even surface charge and structure.[42–44] More important, though, is the study of the opposite effect. Protein adsorption to surfaces is currently known to directly depend on general physicochemical surface properties such as[9,45–49] wettability, chemical composition, roughness, and surface charge energy and tension. Their effect and modification onto protein adsorption and later cell behavior have been extensively analyzed,[50–54] with many researchers persisting on the study of different surface stimuli to optimize the short-term and long-term performance of biomaterials.

The strategy of surface modification of different biomaterials has been adopted over the years in order to alter the area of the biomaterial that first comes in contact with the biological environment. Surface modifications methodologies have been used in a variety of applications for preventing or improving adsorption of proteins and adhesion of cells to biomaterial surfaces.[46,55–64]

Hydrophobicity and hydrophilicity of the surfaces have been extensively exploited. For instance, studies with chitosan[56] show that an increase in hydrophobicity for values of around 100º of water contact angle lead to increased protein adsorption regarding the more hydrophilic nonmodified surfaces. In this case, hydrophobic interactions govern the protein adsorption and the majority of blood proteins form proteinaceous layers over the surfaces.[10,46,56] On the other hand, very hydrophilic surfaces also favor high biocompatibility due to the preferential adsorption of albumin, which firmly binds in high concentration.[10,46] Albumin being highly concentrated and diffusive over the solution medium reaches the surface and binds, leading to a thrombogenicity-lowering effect.[65] In opposition,[54,66] other authors state that strongly hydrophobic or hydrophilic surfaces present a very low ability for protein adsorption.

Other groups of authors state[10,67–69] that the higher water content or higher water uptake ability and minimal interface energy could minimize protein–material interactions and thus decrease the thrombogenic effect. In disagreement, Andrade's work[70] showed that hydroxymethacrylate and methylmethacrylate formulations of high water content presented increased thrombogenicity. Recent studies[71,72] with biodegradable poly(*D,L*-lactic acid) showed a relation between contact angle and surface energy with protein adsorption: preferential albumin and fibronectin was adsorbed onto surfaces of improved hydrophilicity and surface energy.

Polymer surface dynamics and relaxation on a solution environment is exhibited for interfacial free energy minimization: polar components tend to dominate.[73,74] The contact protein surface is function of the chemistry of the polymer in equilibrium with the water and its ions, and with the

protein chemical properties. By contacting a surface, protein dynamics takes place in response to the characteristics found in the interface.[75] Surface physicochemical characteristics such as the surface free energy and electric charge do also affect protein adsorption.[76–78]

22.3 PROTEINS, ADSORPTION, AND KINETICS

The process of protein adsorption onto a solid surface is considered for years as a complex type of interactions of physical and chemical origin that is established between surface, solvent, and proteins.[73,79–89] Characterizing the complexity of protein-covered surfaces is required,[90] demanding advances in surface science instrumentation together with new material science and molecular biology technologies. Finally, a full understanding of the properties of the proteins in study is fundamental for reaching the complexity of the adsorption phenomena.

22.3.1 Assessing Protein Adsorption

The success of protein adsorption studies depends directly on the selected techniques of analysis. Surface science models for application in biological systems are not fully developed. If that was the case, ideally one would be provided with an understanding of how the surface chemistry and structure of a material can be used to control the biological reactivity of a cell interacting with such a surface. To accomplish this goal, understanding cell reactivity and characterizing the complexity of protein-covered surfaces are clearly required.[90]

In the last quarter century, advances in surface science instrumentation together with new material science and molecular biology technologies greatly improved the ability for characterizing interfaces of biological importance. Nevertheless, the majority of the popular techniques are only an approximation of the ideal non-artifact-generating characterization tool. Single protein solutions are generally emphasized over the complex protein mixtures when it comes to the simplicity of the study that must be performed.

The analysis of the amount of proteins that adsorb onto a specific interface must be performed by means of applying highly accurate methods once the amount of adsorbed proteins in function of the surface area is typically very low.[15]

Techniques that give information about the adsorption process and nature of the protein layer are summarized in Table 22.1, together with respective references for allowing the reader to obtain furthermore detailed information. Several techniques have been used to quantify adsorbed proteins, but the preferred strategy is always to combine different techniques in one study, allowing for complementing and adding new information. In the literature, several overviews[1,19,90,91] relate the analysis methods with the variable in study, simultaneously presenting limits and advantages, highlighting new approaches, techniques, and models. The reader can complement the information provided in this chapter with the one that can be found in those studies.

The most traditional technique used over the years for analysis of protein adsorption is the solute depletion technique.[19] In this case, adsorption is determined as the difference between final and initial protein amount following the contact and incubation protein–surface interface. If the obtained result approximates zero, an adsorption rate near to 100% was achieved.[19] This methodology is coupled to other protein detection methods such as: immunoassays,[122] colorimetric,[139] and fluorescence[140] techniques that can also be used for total protein quantification. Simultaneously, radiolabeling has been frequently used[141–143] for determining protein adsorption concentration. This methodology has practical limitations including the easy alteration of the protein affinity for the surface following radiolabeling.[144] For the understanding of the complexity of the molecular aspects of protein adsorption and denaturing, computational chemistry is becoming a very attractive technique.[145,146] Real biomaterials surfaces are still not fully represented, but the contribution given so far by these methodologies may influence the way we look for these phenomena.[146] The authors believe there will be a great future for such type of methodologies.

TABLE 22.1
Methodologies and Techniques Used to Study Different Aspects of Protein Adsorption onto Surfaces

Method	Obtained Information	Ref.
Solute depletion	Adsorbed amount of proteins	92, 93
Direct weighing	Adsorbed amount of proteins	94, 95
Surface plasmon resonance (SPR)	Rate of adsorption and thickness of adsorbed layer	96–98
Ellipsometry	Thickness of adsorbed layer	99–102
X-ray photoelectron spectroscopy (XPS)	Adsorption amount	103, 104
Radiolabeling	Adsorption quantification	105, 106
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and MALDI-MS	Protein adsorption distribution, quantification, composition, and conformation	107–109
Circular dichroism (CD) spectroscopy	Conformation	110, 111
Atomic force microscopy (AFM)	Visualization of conformation	112–114
Confocal microscopy	Detection and conformation of protein adsorption	115, 116
Fourier transform infrared attenuated total internal reflection (FTIR-ATR)	Conformation of adsorption	117–121
Immunoassays	Detection and conformation of adsorbed proteins; adsorption patterns	122–125
Microcalorimetry	Enthalpic changes	126
Raman spectroscopy	Protein conformation	125
Elution method coupled to SDS electrophoresis	Qualitative analysis of protein composition	92, 127, 128
Time-of-flight secondary ion mass spectrometry (ToF SIMS)	Molecular structure	129
Scanning probe microscopy (SPM)	Spatial resolution	130
Near edge x-ray absorption fine structure (NEXAFS)	Chemical specificity	131, 132
Total internal reflection fluorescence (TIRF)	Protein adsorption kinetics, competition, conformation, and lateral mobility	133–135
Axisymmetric drop shape analysis-profile (ADSA-P)	Chronological determination of protein adsorption	136–138

Techniques and their applicability will be further explored along this chapter while related to topics such as different proteins competition to adsorb in a particular surface.

22.3.2 Proteins in Definition

Chemically, proteins are unbranched copolymers of 22 different amino acids of varying hydrophobicity. Some of the *R* groups of amino acids are acidic or basic, conferring to the protein molecules an ambivalent character (see Figure 22.2), and due to their differences in polarity, proteins are rendered surface-active macromolecules of amphiphilic properties.[19,80,147–150] Amino acids are linked by polycondensation, head to tail, from carboxyl group to amino group through the formation of an amine linkage designated peptide bond.[150]

The primary structure of proteins is the polypeptide chain or amino acid sequence. It is important to state that all the information needed for the protein molecule to achieve its architecture is contained within its amino acid sequence.[19,150] The formation of hydrogen bonds between peptide units results in the well-known α-helix or β-sheet, two different noncompact types of secondary structure.[19,80,149] By means of ionic interactions, salt bridges, hydrophobic interactions, hydrogen bonding, and covalent bonds create a more compact structure, called the tertiary structure.[19,80,149] The final possible association is between two polypeptides of organized primary, secondary, and

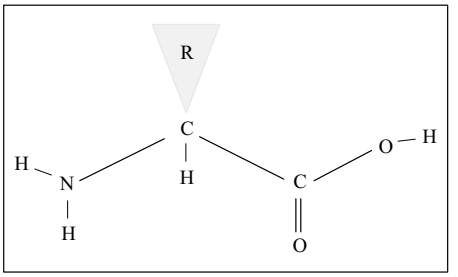

FIGURE 22.2 The amino acid monomer structure. *R* represents the side chain different in each amino acid.

tertiary structures, giving rise to the quaternary structure of proteins.[19,80,147–150] Whereas the primary structure of a protein is determined by covalently linked amino acids residues, other organizational levels are mainly determined by noncovalent forces.[80] More information on protein characteristics can be found in Chapter 1.12.

Any protein adsorption study cannot be useful without first fully understanding the properties of the surfaces or especially while ignoring the properties of the proteins playing the game. Some of the well-studied proteins in the biomaterials field are[151–153] serum albumin, fibronectin, and vitronectin.

Albumin is the most abundant protein in the human blood serum and due to its concentration and ability to bind other molecules is seriously considered as a model protein also in terms of protein competition.[154–156] More specifically, albumin's primary role is the transport of fatty acids, but it is also responsible for the maintenance of colloidal osmotic blood pressure and detoxification.[154–158] Human serum albumin is a heart-shaped and monomeric protein composed by 585 amino acids that compose a total of 66,400 Da of molecular weight.[155,158]

Most of the proteins contain short carbohydrate sequences and are therefore called glycoproteins.[19,150] Fibronectin (FN) is a large glycoprotein formed by two disulfide bounded polypeptides that can be found in blood plasma and other fluids on its soluble form, but also as structural protein on solid tissues.[159–161] FN is an extended molecule folded in globular domains of particular functions and linear arrangement of repeating units of amino acids, know as type I, II, and III.[162,163] Several research studies[164–168] have proved the influence of this molecule in interacting with integrin and nonintegrin cell surface receptors, through which cell adhesion, migration, proliferation, and differentiation are affected. In central location of the chain, type II repeats were identified to include both arginine-glycine-aspartic acid (RGD) motif and the PHSRN (proline-histidine-serine-arginine-asparagine) synergistic sequence.[162,169]

Vitronectin, also found in plasma and ECM, is a multifunctional glycoprotein of approximately 75 kDa, which comprises the important RGD peptide that is known for mediating attachment and spreading of cells.[170] On the other hand, by binding to plasminogen activation inhibitor-1,[171] this protein can potentially regulate the proteolytic degradation of the extracellular matrix, and is also involved in the immune response and clot formation.[107,172]

22.3.3 Adsorption and Desorption Kinetics

In an adsorption study, several parameters are always to be considered. In Figure 22.3, a simplistic schematic representation of several factors affecting the phenomena of protein adsorption illustrates the tri-element situation: surface-solution-protein. Concerning surface characteristics, some of the fundamental aspects are[19,147,173–176] surface hydrophobic nature, topography, heterogeneity, surface composition, chemistry, interfacial dynamics, and surface stability in water. On the other hand, protein characteristics as the isoelectric point, charge distribution, three-dimensional (3-D) conformation and stability, the distribution and nature of hydrophobic domains, and the ability to bind low-molecular-weight species need to be considered. [19,176] The pH, ionic strength, ionic compounds,

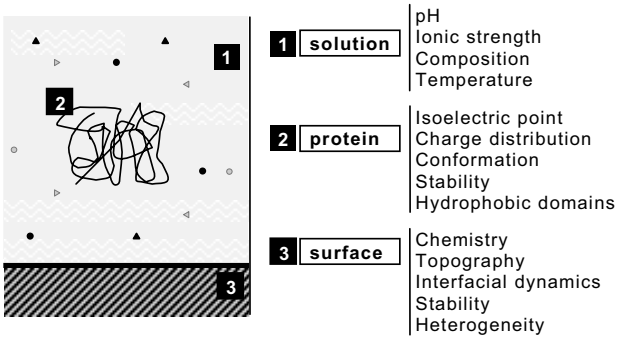

FIGURE 22.3 Simplistic cartoon representing some of the solution, protein, and surface factors influent in the protein adsorption event.

the buffer nature, the presence of low-molecular-weight species, and also the solutions temperature, pressure, or hydrodynamic flow are decisive in terms of the final protein–surface interactions.[19,176–179]

The adsorption of proteins at interfaces has been shown to be a complex phenomenon that includes the diffusion of the protein species through an aqueous medium and its collision and interaction at the interface,[180] where the major driving factor is the ensuing entropy gain.[80] Several papers[80,146,147,181–186] are available in the bibliography where models of the thermodynamics of adsorption isotherms are described.

Over the last two decades, several authors proposed models to explain the adsorption phenomena. Examples are Beissinger and Leonard,[187] Soderquist and Walton,[188] and Lundström and Elwing.[189] The complexity of this last model already includes the concepts of[189] adsorption constant k_a, desorption constant k_d, and conformational changes and exchange constants, k_e and k_c, respectively. According to Norde,[80] the process of adsorption kinetics is typically divided in five steps: transport to the surface; adsorption; time-dependent rearrangement; desorption or exchange; and diffusion away from the surface. Measuring the protein–surface interaction is one of the major goals in this field, translating the affinity of a certain protein to a surface. In 1986, Horbett and Brash[180] proposed that this affinity phenomenon could be deduced from kinetic observations under diffusion limit, which would directly lead to the sticking coefficient. According to the authors, the sticking coefficient reflects the number of collisions that lead to adsorption being a function of the molecular interactions between the protein and the surface.[180,190] The study of the sticking coefficient has been intimately discussed with another concept, the *elastic barrier*.[180,190] Within this concept, a certain protein in solution and approaching the surface has probability to adsorb and 1 – to be reflected. Also considered is the *random walk* of this particle, or in other words, the *journey* performed by the molecule in the proximity of the surface.[180,190]

Several different studies[144,191] have been reporting the difficulties and practical limitations of the estimation of protein adsorption onto polymeric surfaces. One of the prerequisites for the analysis of the biological reactivity of a material is the kinetic association constant of high information value over the early adsorption times.[191] The difficulty for achieving this quantitative element regards the complexity of the determination of the adsorbed proteins over the first seconds of adsorption. In this case, several techniques can be used, but the most successful one has been reported to be ellipsometry.[54,101,192–195] Also, the steric hindrance and mass transport considerations hinder the accurate determination of k_a.[19,47,191,196] The interaction of the water components with the surface generates potential adsorption sites and the balance between protein concentration, surface area, and volume for diffusion make experiments a difficult issue to control. Another of the difficulties is when surfaces come in contact with the air, the protein layer will denature by conformational changes, which explains the air avoiding need of the techniques and models developed on the scope of the kinetics studies.[43] A spontaneous structural arrangement is expected

when a protein molecule in solution touches a solid surface. Binding and orientation of proteins is one of the most studied topics of the protein adsorption issue.[197]

Desorption of proteins from surfaces has been reported to be nonexistent or very slow, which in other words means irreversible, partially reversible,[80,198] and also reversible process.[199] pH changes and ionic strength can completely remove molecules from surfaces, which are often used for protein adsorption analysis methods, such as 2-D electrophoresis.[1,19,200,201] New ionic strength conditions allow for bound proteins to be eluted from the surface into the solution in a more pronounced way for hydrophilic surfaces than for hydrophobic ones.[19,80,176,198,202–204] The discussion of desorption mechanisms cannot be dissociated from two other concepts: exchange and protein competition, both characterized as a high speed interfacial phenomena.[194,205–208]

22.4 EXCHANGE AND COMPETITION OF PROTEINS: BLOOD PLASMA AND COMPLEX SOLUTIONS

The complexity of the issue protein adsorption was very well reviewed by Andrade et al.[209] using a axis concept, which ranges from relatively simple proteins to the very complex ones following the multi-component protein solutions such as blood plasma and tears.

By means of simply performing single protein adsorption studies, neither the complexity of blood bioenvironment nor the biocompatible potential of biomaterial surfaces can be assessed.[96,210] When it comes to the adsorption from blood, plasma surfaces are enriched on a number of protein species. The limited number of adsorption sites per unit area drives to a selection process regulated by the intrinsic ability of some plasma proteins for preferential adsorption in opposition to others.[1,134,211,212] In this sense, competition phenomena can only be measured by means of studying multi-protein solutions such as blood plasma[96,122,213,214] or mixtures of plasma proteins.[96,108,122,215]

Essentially, all the biological fluids are multi-protein systems. Blood plasma was first studied by Vroman and Adams in 1969.[216] Proteins were suggested to adsorb sequentially starting from the abundant low-molecular-weight ones, like serum albumin, and ending, for longer time periods and after species exchange, with kininogen as preferentially adsorbed onto the formed layer.[216] These experimental findings gave rise to the so-called Vroman effect.[8,217–223] More specifically, after the contact of blood or plasma with crystal and glass modified surfaces, the absorbate composition changes with time as a result of the consecutive replacement of the adsorbed proteins. In the early stages, smaller and higher concentrated protein species will reach easily the surface for adsorption, being later exchanged by higher surface activity ones and less concentrated proteins. According to their observations, Vroman and Adams[8,220,221,224–226] proposed the following sequence of adsorption onto blood contacting surfaces: albumin, immunoglobulins, fibrinogen, fibronectin, and high-molecular-weight kininogen and factor XII.

The effect of concentration coupled to the residence time was found to modulate the amount and composition of the protein layer.[96,215] Although these observations became generalized for several proteins and surfaces, systems were observed to be excluded from this concept.[217,227] Both the degree of dilution[227] and the type of surface[228] affect the kinetics and sequence of exchange, thus influencing the absorbate composition. Besides residence time and surface properties, the protein nature, unfolding rate, diffusion constants, surface affinity, or ability for irreversible binding will determine the conformational change and interaction with the surface to achieve the most favorable energetic state.[134,227,229]

Considering this, competition and exchange of proteins cannot be understood as separate mechanisms. It is frequently observed that proteins desorb into solution at a very low rate, in opposition to the situation where new or other protein species are present.[134] In the early 1980s, Jennisen proposed a molecular explanation for these observations.[230] Proteins adsorb, forming multiple contact points with the surface, which are unlikely to disappear at the same time in accordance to the observed low spontaneous desorption rate. If other more active proteins start

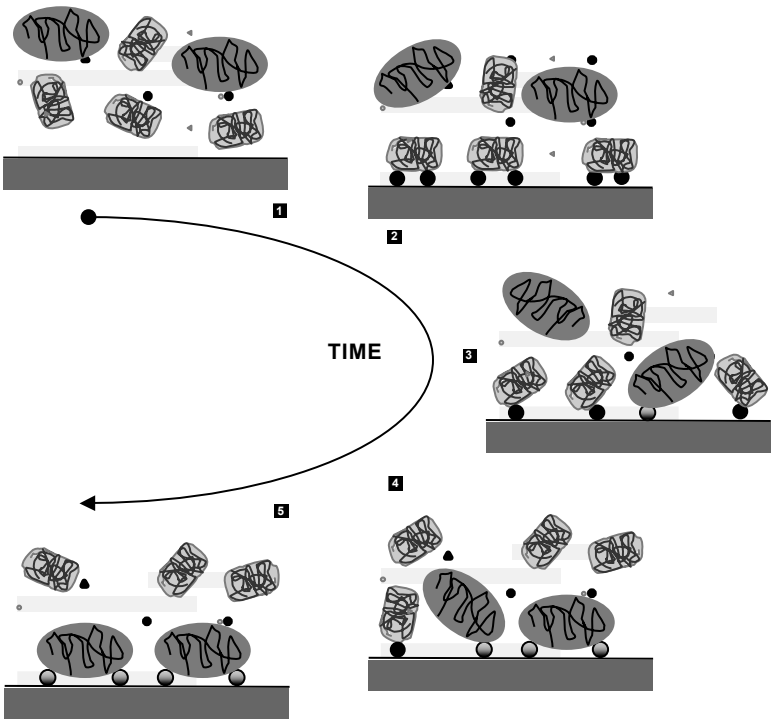

FIGURE 22.4 Simplistic cartoon representing the exchange and competition of proteins from the earliest to the later stages. Concentrated small proteins with higher diffusion constants (light gray shading) and lower concentrated proteins with higher dimension and binding affinity (dark gray shading) are symbolized as previously indicated.

adsorbing in these newly created free spaces, eventually the old protein will be replaced by the establishment of contact points between the new protein and the surface (Figure 22.4). Thus, the increasing desorption rate could be promoted by protein exchange.[230]

Protein competition is expected to happen simultaneously to the adsorption of the molecules to a presented surface. Competition between proteins is conditioned by several parameters[205,231] such as diffusion coefficient, molecular mass, polarity, or electrical charge of the proteins in a determined evolving fluid. Surface properties are known to greatly affect adsorption of single-protein systems.[154,203]

Although inappropriate for simulating the bioenvironment complexity, attempts[1,19] have been made to find general rules for relating the different protein characteristics found throughout single protein solutions, to the adsorption in competitive environment. Still in this context and on the biotechnological level, the development of new techniques to evaluate complex protein solutions in contact with the material surfaces is becoming urgent.

Exchange reaction models have been proposed for the analysis of complex protein solutions,[228] and several techniques have been adapted for this application. The most common ones are radio-labeling,[232–234] fluorescence,[235] and ellipsometry.[236,237] More recently, methodologies such as surface plasmon resonance[96] and x-ray photoelectron spectroscopy (XPS) coupled to surface matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy[108,238] have been applied. Also, methodologies based in antibody-specific binding properties, such as the enzyme linked immunoassay (ELISA) system, have been selected and applied by several authors.[122,239–241]

Norde and Lyklema showed that the stability/rearrangement ability of the proteins strongly affects preferential adsorption of less stable proteins in favor of the most stable ones.[202]

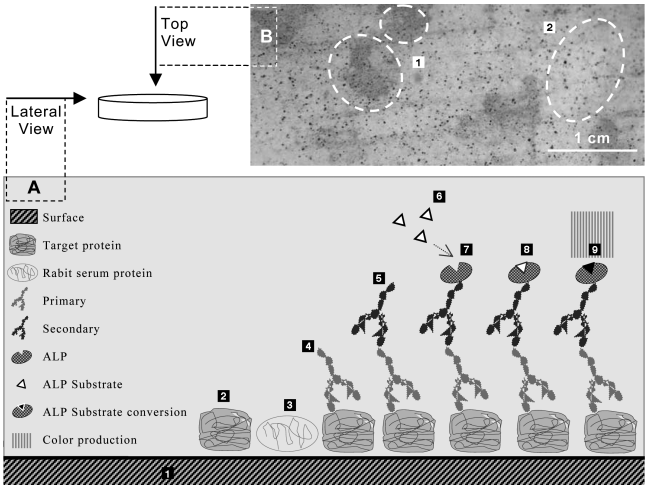

FIGURE 22.5 The immunoassay methodology in a simplistic sequence cartoon (A) simulating a lateral view and the final result for a starch-based material surface previously coated with human fibronectin and labeled with anti-FN (B).

Lassen and Malmsten[215] have shown in 1997 that human serum albumin (HSA), immunoglobulin (IgG), and fibrinogen (Fgn) extensively adsorbed onto both hydrophilic surfaces of different charges, but negatively charged ones evidenced simultaneously, slower adsorption kinetics, and HSA exchange under the presence of other proteins. Regarding protein competition, Fgn was predominantly adsorbed on both these surfaces while for a third hydrophobic surface, IgG and albumin dominated the protein layer.[215] Earlier on, the same authors demonstrated the ability of albumin for blocking other proteins adsorption on the presence of hydrophobic polymer surfaces due to irreversible adsorption associated to conformational changes of albumin.[242] In another study,[233] adsorption of collagen was observed reduced in the presence of albumin and to dramatically decrease with the increase of surface hydrophobicity.[233]

The use of antibodies to label human proteins allows for obtaining reproducible and useful results for understanding protein adsorption on biodegradable surfaces (Figure 22.5).[122] Immunoassays are aimed to detect a specific target protein (Figure 22.5A, step 2), using blocking proteins (Figure 22.5A, step 3) for minimizing the nonspecific binding of the primary antibody (Figure 22.5A, step 4) to the surface (Figure 22.5A, step 1). To this antitarget protein antibody specifically binds the secondary, which in turn binds alkaline phosphatase (ALP). In the presence of the substrate, enzyme catalysis takes place and a different color is produced on the surface (Figure 22.5A, steps 5, 6, 7, 8, and 9, respectively). In Figure 22.5B, labels 1 and 2 show color production and absence, respectively, onto the surface of a starch and cellulose acetate (SCA) polymeric blend reinforced with hydroxylapatite. Antibody labeling technique has been used with different polymeric blends of starch with cellulose acetate (SCA), ethylene vinyl alcohol (SEVA-C), and polycaprolactone (SPCL).[122] Besides the synthetic phase being different for each material, the percentage of starch is also variable: 50% for SCA and SEVA-C and 30% for SPCL starch-based blends. Single, binary, and serum diluted solutions were prepared using human sources and the proportion of the proteins in the human blood serum was considered.[154] Results allowed to observe that after 24 hours, fibronectin (FN) and vitronectin (VN) adsorbed in higher amounts than the more concentrated HSA.[122] When studying binary systems, FN in the presence of HSA was found to adsorb less than albumin on the SEVA-C surface, in opposition to the observations obtained for single protein solutions. Both the chemistry of the material and the presence of other proteins were found to be determinant for the final adsorbed layer. When diluted human blood serum was used as the protein source, differences in protein adsorbate after 24 hours of incubation were observed.[122] As

a general trend, the competitive potential of albumin to adsorb onto starch-based surfaces was decreased.[122] This can be related to the lower activity, size, higher concentration of this protein, and also with the residence time.[96,154–156,215] In agreement, vitronectin and to some extent fibronectin were the most highly adsorbed proteins independent of the surface used. In terms of polymeric blend types, it was also found that cornstarch with polycaprolactone presented the highest protein adsorption levels independent of the protein species.

These results show the influence of the polymer nature and the protein specie onto the final adsorbate layer.[122] Furthermore, these results agree with earlier performed experiments of Fabrizius-Homan and Cooper,[233] which showed that following the contact of polymeric surfaces with diluted serum, plasmatic proteins do adsorb, but a significant enrichment of vitronectin is observed in the surface.

22.5 PROTEIN RECOGNITION AND CELL ADHESION MECHANISMS

The recognition that cellular functions at the implant-interface are determinant for the degree of success of a biomedical device, the clinical use of a material which allows for predicting and to develop beneficial reactions from the implants surrounding cells and tissue, can be easily understood.

In the early 1990s, Ratner[243] envisioned and described for the first time the "next generation of biomaterials" as engineered surfaces deliberated to invoke specific cell responses. Furthermore, this concept would include materials able to compensate the complex medical condition of the injured patient, simultaneously improving the healing process.[243]

For the design of biomaterial devices, normal cell mechanisms such as adhesion, migration, proliferation, and differentiation of the specific cell populations involved are clearly required to be understood.[244]

Anchorage-dependent cells such as fibroblasts, osteoblasts, or endothelial cells are so designated due to the need of establishing adhesion mechanisms for normal cell functioning as development, organization, and maintenance of tissues.[245,246] Following attachment, an intracellular cascade of events will be developed, leading to the regular phenotype and genotype development of the specific cell lineage, including spreading, differentiation, secretion, extracellular matrix production, and migration. Adhesive cells use extracellular matrix proteins (ECM) to attach and to migrate on substrates.

The primary mode of adhesion and migration is performed by means of integrins, cell transmembranar receptors composed by α and β units that recognize the well-studied RGD peptides.[247–250] Integrins proteins are expressed in several cell types including bone and in bone cells culture.[251] RGD adhesive sequences, so designated due to its ability to bind a specific ligand,[252] are known to be present in several ECM proteins including bone sialoprotein, collagen, fibronectin, osteopontin, thrombospondin, and vitronectin.[253–256] Following the receptor–sequence interaction, integrins cluster together and organize into focal adhesion complexes with mechanical and chemical activity of cell anchoring, generating an intracellular cascade of multiple signaling events.[250,257–259] This subsequently regulates cell migration, proliferation, phenotype, genotype, and thus, cell differentiation.[250,257,260–262] In this context, the behavior of anchorage-dependent cells seems to depend on the availability/exposition of these adhesion sequences, which, in turn, is affected by the protein three-dimensional conformation. Adhesion of a cell to the underlying substratum can be controlled by increasing the ligand density, the affinity of the binding receptor-ligand, or by the amount of adhesion receptors expressed on the exterior surface of the cell phospholipids membrane.[263] Several integrins need other peptidic sequences for efficient binding. For instance, $\alpha_5\beta_1$ integrin involved in the control of osteoblast and myoblast cells proliferation and differentiation[261,264] only binds to RGD segment on the tenth type III repeat of fibronectin in the presence of the PHSRN motif (proline-histidine-serine-arginine-asparagine) to which binding is performed with ninth type III repeat. Adhesion strength is significantly increased by this synergistic association.[265,266]

Intimately connected to the adhesion of cells is the process by which cell movement is triggered.[267–269] Cell migration is a complex dynamic mechanism achieved by the cell transition of cytoplasmic generated forces into tractional forces that will pull the cell itself across the substrate.[270,271] Traction is provided by the interaction between integrins, cell adhesion surface receptors, and specific ligands covalently immobilized on the biomaterial surface.[249,272,273] Following these receptor–ligand interactions, several intracellular processes take place, namely, cytoskeleton organization, signaling, force generation, cell body displacement, and rupture of the bonding receptor–ligand in the surface area opposite to migration direction.[274–277] That is, migrating cells, while protruding and stabilizing leading edges, release the early formed complexes at the rear of the cell.[278] The extent or strength of the adhesion between the cell and the underlying extracellular matrix (ECM) is critical in determining the efficiency of cell migration.[263,272] Experimental work developed by Dee et al.[268] studied the dependence of random migration, also designated as haptokinesis onto RGDS and RDGS peptides, which include adhesive and nonadhesive domains, respectively. Results showed significant reduction of migration and enhanced proliferation of osteoblasts over RGDS segments as compared to RDGS-modified surfaces.[268] Thus, the correlation between migration and proliferation, and the contribution of both processes on the surface colonization by osteoblastic cells was shown. It is rather important to realize that migration has an important function in processes such as embryogenesis, inflammation, and tumurogenesis[270] and in the colonization of newly body-contacting surfaces as the desired migration of bone-related cells to the implanted bone prosthesis.[268]

22.6 SELECTIVE PROTEIN ADSORPTION: STRATEGIES FOR CONTROLLING AND MODULATING CELL AND TISSUE RESPONSE

Several literature sources[5,9,279] describe the problem of nonspecific bioadhesion, such as protein adsorption, as the main cause of biomaterials failure due to uncontrolled accumulation of biological material at the interface. Controlling this nonspecific phenomenon requires the manipulation of the chemical, physical, and biochemical properties of an implant surface. Underneath the biomaterials scope, it is widely recognized that the first interaction between biomedical devices and the biological environment occurs at the interface, which plays an important role in the biomaterials design. Attempts have been made[146,280–284] in order to control the adsorption of proteins mainly by means of surface modification methodologies as the use of polysaccharides, phospholipids, proteins, fragments, and grafting of polymer molecules to the surface of the materials.

For the control of protein adsorption and biomaterials design, surface treatments, different protein species, their concentration and physical properties of the aqueous environment (blood or tears) are to be considered. Moreover, the chemistry and physics of the surface and the application timescale of the device play important roles.

Designing surfaces to control and predict protein adsorption is not a new topic. In this context, during the last 20 years, researchers have conducted experimental and theoretical work[73,209,285] that resulted in a lot of information but low molecular-based comprehension. The understanding of submolecular events of proteins adsorption is fundamental and the emerging of even more powerful characterization techniques is becoming urgent.[146]

22.6.1 Proteins and Peptide Sequences

The identification and recognition of the value of adhesive sequences (such as RGD and PHSRN) motivated bio-inspired surface modification techniques as the incorporation of short peptides onto adequate surfaces, which are generally nonadhesive. The ultimate goal of these protein-mimetic surfaces is the reduction of nonspecific protein adsorption for obtaining functional surfaces.[286]

In the last years, several groups have been studying, both *in vitro* and *in vivo*, different surfaces incorporated with cell adhesion peptides on the binding properties, attachment, proliferation, differentiation, migration, morphology, and spreading of cell lines and primary cultures[197,273,287–293] (see Table 22.2). These approaches offer advantages over the use of entire molecules including[294–296] decreased antigenicity and increased biocompatibility by removing domains capable of starting adverse reactions, such as complement activation domains, fibrinogen, collagen, and heparin-binding domains. Simultaneously, recombinant fragments allow for conferring specific characteristics for enhancing the immobilization of proteins improving their activity and finally the ratio efficiency/financial cost increases.[297] On the other hand, according to Akiyama et al.,[298] the use of small segments instead of higher dimension fragments increases the possibility of defect in activity due to conformational changes and also, represents lack of integrin specificity.

The use of complete proteins instead of small peptide segments or sequences envisions the study of *in vivo* biology by simulating the molecular structures presented by nature.

Several studies reveal the cellular effect of preadsorbing different proteins onto polymer surfaces. Fibronectin was soon considered the archetypal cell-adhesive protein[256,305] and a regulator of cell behavior,[163,306] generating research interest in studying the effect of this molecule over different polymeric surfaces and in terms of cell phenotype and behavior observation and control: adhesion and spreading,[259,307–314] migration,[267,315,316] proliferation,[261,317] and signaling pathways.[258,259] Besides fibronectin, other proteins such as fibrinogen,[311,318–321] albumin,[240,319,322,323] and collagens[312,324,325] have also been extensively studied.

22.6.2 Protein-Resistant Surfaces

In the case of blood-contacting biomaterials, the adsorption of plasma proteins is known to occur within seconds of exposure and to trigger numerous adverse effects: coagulation, platelet adhesion and activation, complement activation, and immunological reactions such as thrombosis.[213,326–329] By compromising the normal homeostasis of the particular bioenvironment, the implant can be subjected to biological reactions somehow similar to body response to virus attack response but an increased scale. Depending on its composition, the protein layer can trigger adverse biological mechanisms: certain concentration of platelet-adhesive proteins, such as fibrinogen and fibronectin, develops mural aggregates and thrombus. Thrombus formation may block smaller-diameter vascular grafts and embolization, leading further on to more serious complications. Besides hemocompatibility-related devices, on ophthalmic applications, the adsorption of tear proteins is associated with lens fouling. Similarly, the applicability and usage of lenses becomes limited and determinant of patient discomfort.[330] Also undesirable is the protein adsorption effect on accelerating clearing of bare liposomes by the reticuloendothelial system.[331]

The preexisting know-how on thrombogenesis, foreign-body response, and interfacial protein behavior as natural biological mechanisms of body defense allowed for the development of approaches for its prevention: protein-resistant surfaces.

To obtain a nonfouling surface, the combined forces of attraction, such as van der Waals, electrostatic, entropic, and hydrogen-bonding forces, the protein surface needs to be smaller than the entropic and hydrodynamic repulsion due to thermal motions of the flexible molecular chains and solvent molecules.

Recent work[332–334] showed how the ability of a polymer layer in reducing protein adsorption, either kinetically or thermodynamically, largely depends on the surface coverage of grafted polymer and in the interaction of these segments and the surface. When the polymer chains are attracted to the surface, proteins in the surroundings are subject to a strong steric repulsion and also competition for the surface between proteins and polymer chains will take place. Both these aspects explain why surfaces that attract the polymer show lower protein adsorption than surfaces that do not, although an equilibrium is needed for assuring the formation of an efficient long-range steric barrier for proteins.[280]

TABLE 22.2
Different Protein Peptide Domains and Peptide Combinations Found to Control Cell Response

Peptide and Amino Acid Composition	Type of Domain	Observed Cellular Effect	Ref.
RGD (arginine-glycine-aspartic acid)	Adhesive	Neonatal rat calvarial osteoblasts presented enhanced attachment, spreading, and cytoskeleton organization. The formation of mineralized matrix was stimulated.	299
YIGSRG (tyrosine-isoleucine-glycine-serine-arginine-glycine)	Adhesive	Enhanced bovine endothelial cells proliferation and motility	244
CRGD (cysteine-arginine-glycine-aspartic acid), CREDV (cysteine-arginine-glutamate-aspartic acid -valine), and CCRRGDWLC (cysteine-cysteine-arginine-arginine-glycine-aspartic acid-tryptophan-leucine-cysteine)	Adhesive	CCRRGDWLC enhanced human vascular endothelial cells adhesion and mouse fibroblasts adhered best to CREDV.	300
RGDS (RGD-serine)	Adhesive	Enhanced mouse fibroblast cells proliferation	301
RGDS (RGD-serine) and RDGS[a] (arginine-aspartic acid-glycine-serine)	Adhesive and nonadhesive[a]	Reduction of haptokinesis of neonatal rat calvarial osteoblasts decreased on adhesive peptide presence.	268
GRGDSPC (glycine-RGD-serine-proline-cysteine) and GRGESPC (glycine-arginine-glycine-glutamic acid-serine-proline-cysteine)	Adhesive and nonadhesive[a]	Mouse melanoma cells migration persistence time decreased for increasing adhesiveness.	272
RGD and PHSRN (proline-histidine-serine-arginine-asparagine)	Both adhesive, and PHSRN is a FN synergy site	Macrophage adhesion, activation, and foreign-body giant cells formation (FBGC)	302
RGD, PHSRN, and PRRARV (praline-arginine-arginine-alanine-arginine-valine)	All adhesive, and PRRARV is a FN C-terminal heparin-binding domain	RGD and PHSRN promoted FBGC in opposition to PRRARV.	303
RGDS, YIGSR, VAPG (valine-alanine-proline-glycine), VGVAPG (valine-glycine-VAPG), KQAGDV (lysine-glutamine-alanine-glycine-aspartic acid-valine), and RGES (arginine-glycine-glutamic acid-serine)	All adhesive and RGES nonadhesive[a]	Adhesion of SHR smooth muscle cells increased for all adhesion peptides.	304
RGD, PHSRN, and PRRARV (praline-arginine-arginine-alanine-arginine-valine)	All adhesive and PRRARV is a FN C-terminal heparin-binding domain	RGD and PHSRN promoted FBGC in opposition to PRRARV.	303
FNIII7-10 (FN fragment compassing RGD and PHSRN domains)	Both adhesive	Murine immature osteoblast-like cells adhered via a5b1 integrins, spread, displayed cytoskeleton reorganization, and assembled robust focal adhesions.	297

[a] Used as negative controls.

Such type of surfaces can be achieved by means of immobilizing neutral and hydrophilic polymers such as poly(ethylene oxide) (PEO).[335–337] In fact, early and recent studies of protein-resistant surfaces have been mainly motivated with PEO and its low-molecular-weight equivalent, and poly(ethylene glycol) (PEG; $M_w < 10{,}000$).[338–340] The interactions between PEO and proteins have been widely investigated and modeled. Poly(ethylene oxide) is a neutral polymer soluble in aqueous media because of the formation of hydrogen bonds with the water. Within this system description, PEO is a simple polymer while comprising monomers that exist in a single start and a complex polymer due to the number of different interconverting states in which the monomer can exist.[331]

PEO polymers exhibit the minimum of interfacial free energy considering water-soluble polymers. Both polymers are hydrophilic, presenting unique properties and molecular conformation in water, and exhibit high surface mobility and steric stabilization.[339,341–343] In opposition, these polymers are also soluble in organic solvents, a result of their hydrophobicity. This conjugation is a distinguishable property fundamental for these polymers excellent biocompatibility. The mechanisms responsible for protein adsorption reduction and eliminating nonspecific adsorption are not fully understood. According to several authors,[49,344] PEO surface density and molecular weight are the decisive characteristics that enable these polymers for protein resistance. In the literature, several techniques are described as adequate to generate PEO-rich surfaces: physical coating, chemical coupling, and graft copolymerization,[316,330,341,345–347] by which several types of surfaces have been modified on the scope of different biomedical application.[56,323,330,341,348–351]

Other polymers have been indicated in the literature as low protein interacting surfaces: 2-methacryloyloxyethyl phosphorylcholine[352,353] and other methacrylates with phosphorylcholine group,[354] poly(*L*-lysine)-graft-poly(ethylene glycol),[355] poly(acrylamide), poly(*N,N*-dimethylacrylamide), poly(vinyl alcohol), and ethylene–vinyl alcohol copolymer.[343] The effect of coupling proteins, protein residues, and other molecules has also been described.[356,357] Examples are albumin,[358] hirudin,[359] thrombomodulin,[360] He^+ ion implanted collagen,[350] and variable saccharides such as maltose, maltoheptaose, and oligomaltose.[356,357] By means of using substrates able to resist to cells adhesion, likewise PEG hydrogels are considered for years,[336] coupled to cell selective ligands allow for binding a specific cell type. On this knowledge, nonadhesive scaffolds have been incorporated with ligands for selective cell adhesion.[361–364] Hubbell and coworkers used fibronectin-derived REDV (arginine-glutamine-aspartic acid-valine) peptides onto non-cell-adhesive biomaterials showing the exclusive adhesion of endothelial cells and resistance to fibroblasts, smooth muscle cells, and platelets.[365] Also, Mann et al.[304,366] successfully photopolymerized TGFβ-1 (transforming growth factor beta-1) to hydrogel surfaces, which led to improved material mechanical properties and increased collagen synthesis, thus improving cell biological activities for certain biomaterial applications. It is recognized that the adhesion and proliferation of different types of cells on polymeric materials depend on different surface characteristics,[367] as it has been demonstrated that cell adhesion occurred preferentially to water wettable substrates.[368] Starch-based blends (with ethylene vinyl alcohol [50/50 wt%], SEVA-C; with cellulose acetate [50/50 wt%], SCA; and with polycaprolactone [30/70 wt%], SPCL) have been surface modified in order to enhance cell adhesion and proliferation on their surfaces. Two different methods have been used – chemical surface modification by potassium permanganate/nitric acid system[369] and surface modification by UV-irradiation.[370] In general, both surface treatments have resulted in higher oxygen content (XPS) and as a consequence in lower water contact angle values. This resulted in an increase of the number of human osteosarcoma cell SaOs-2 onto the modified surfaces, especially higher for the blend with polycaprolactone.

22.7 FUTURE DIRECTIONS AND CONCLUDING REMARKS

The interdisciplinary nature of protein adsorption studies defines a complex field of research, where the drawbacks of current applied techniques limit the perfect understanding of the sensitive

protein adsorption microenvironment. Efforts, mainly carried out in recent decades, accomplished the present know-how, but several voids are still to be filled. Nevertheless, these works allowed for reaching a point where surface manipulation for different biomedical applications cannot typically go further before studying the characteristics of the protein adsorbate. This also explains the development and motivation for applying protein-related molecules to different surfaces. These types of studies are carried out in several of the most prominent groups that work on the biomaterials field.

After detecting what is desirable or undesirable in terms of the proteins behavior over biomaterial surfaces, the motivation for its control is now the state of the art and the goal in this field. The idea is not to disregard cells or cellular structures but to aim exactly for directing cell phenotype and implant success improvement. In this sense, there is still a gap between the protein adsorption behavior observed in *in vitro* situations and the respective correlation with what happens in the *in vivo* bioenvironment.

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23 Surface Activation and Modification — A Way for Improving the Biocompatibility of Degradable Biomaterials

I. Pashkuleva and Rui L. Reis

CONTENTS

23.1 INTRODUCTION

The communication of an implant with the host system takes place initially via the surface. This initial, direct contact between the living tissues in the body and the surface is a major factor that determines the rejection or acceptance of a foreign device. In general terms, the ideal biomaterial should have adequate mechanical (and other) properties while the surface should have good biocompatibility, i.e., a material must possess not only suitable mechanical properties in order to function properly in a bioenvironment, but should also not be harmful for the host tissue and if possible, should not induce any inflammatory response. For instance, a material for hip joint has to be able to stand high stress and at the same time, cells should proliferate on its surface. In other cases — for example, for catheters — the requirements are opposite; the material is considered to be compatible if it is flexible enough and prohibits cell growth. However, it is rare that a biomaterial with good bulk properties also possesses the surface characteristics suitable for clinical application, and very few surfaces are truly biocompatible. This has been discussed in a review by Chu et al.[1] Therefore, the only possibility for men is to "dupe" the host. One common approach is to fabricate biomaterials with adequate bulk properties and then to "make up" those by a special treatment resulting in enhanced surface properties.

The surface behavior is dependent on many parameters. Unfortunately, one cannot yet specify which of them are most important for understanding biological responses to surfaces. Studies have been published[2–12] on the importance of roughness, wettability, surface mobility, chemical composition, crystallinity, and heterogeneity to biological reaction. Since one cannot be certain which surface factors are predominant in each situation, the controlling variable or variables must be independently ascertained.[12,13]

How one can change and control the certain surface parameters and to make a biomaterial to behave according to one's expectation? Similar to the people faces, materials surfaces are very different and it is not possible to have a universal modification for all of them. Moreover, the environment and the role that a certain biomaterial is expected to play call for special, unique, and sufficiently resistant "makeups" that must ensure its good performance. The depth of the performed modification is another key property related with this. If the modified layer is very thick, there is a risk to change the mechanical and functional properties of the material or to delaminate it (if it is a coating) because of mismatching with the substrate in physical properties. On the other hand, very thin layers are not favorable and their resistance as well as activity are doubtful. For biomaterial surface modification, it was found that layers with a thickness of some ten to hundred nanometers should be sufficient.[14]

There are a lot of different classifications of surface modification methods: according to the forming bond type (covalent or noncovalent), the used source (plasma, UV, etc.), and the mechanism (whether the modification is on the existing surface [plasma treatment, etching, chemical modification, etc.] or whether it overcoats the existing surface [covalently or noncovalently attached] with a material having a different composition [coating, grafting, thin film deposition, etc.]).

Most of the typically used surface modification methods are presented in Table 23.1,[15] but not all of them will be discussed in the present chapter.

23.1.1 Physical Modifications

The activities aimed at physically modifying polymeric surfaces can be divided into two main categories, the first involved with chemically altering the surface layer, the second with depositing an extraneous layer on top of the existing material, thereby generating a sharp interface.[16]

23.2 PLASMA SURFACE MODIFICATION OF BIOMATERIALS

Plasma surface modification methods have some common advantages that can be generically listed as follows:

TABLE 23.1
Surface Modification Methods

Modification of the Original Surface
Plasma etching
Plasma implantation
Corona discharge
Electron beam treatment
UV irradiation
Chemical reaction
Nonspecific oxidation
Functional group modification
Addition reactions
Conversion coatings
Covalently Attached Coatings
Grafting with ionization radiation
Photografting (UV and visible sources)
Grafting with pretreated polymer surfaces
Plasma polymerization
Plasma treatment (gas discharge) (RF, microwave, acoustic)
Gas phase deposition
Ion beam sputtering
Chemical vapor deposition
Chemical grafting
Biological modification (biomolecule immobilization)
Noncovalent Coatings
Langmuir–Blodgett film deposition
Self-assembled layers
Surface active additives
Plasma vapor deposition (PVD)

Data from Ratner, B.D., *Biosens. Bioelectron.*, 10, 797, 1995.

Excited species in a gas plasma can modify the surfaces of all polymers, regardless of their structures and chemical reactivity.
By choice of the gas used, it is possible to choose the type of chemical modification for the polymer surface.
The use of gas plasma can avoid the problems encountered in wet chemical techniques (residual solvent on the surface, swelling, etc.).
Modification is fairly uniform over the whole surface.

This type of technique presents some disadvantages as well:

Plasma treatments must be carried out in vacuum.
The optimal parameters for one system usually cannot be adopted for another system.
It is very difficult to control precisely the amount of a particular function group formed on the surface.

23.2.1 Some Terms

The *plasma* is nothing more than a "tank" of various energetic and reactive species[17] and could be considered as the fourth state of the matter (Figure 23.1), which is composed of highly excited atomic, molecular, ionic, and radical species.

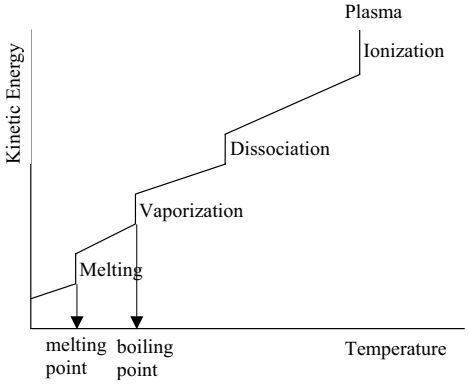

FIGURE 23.1 Transitional states of the matter.

Despite containing positively and negatively charged particles, the whole of plasma is neutral. Further, the plasma state does not continue at atmospheric pressure, but at a low pressure of 1×10^{-2} Torr. To create and sustain plasma, energy is required to keep up to the *ionization rate*. The magnitude of the *energy for ionization* is equivalent to that of carrying away an electron in an atomic nucleus toward an infinite distance. This energy is supplied by an external electrical field. Different *plasma sources* can be used — gaseous (radio frequency glow discharge and corona discharge), metallic, and laser based.[1,14,18] A plasma reactor typically consists of a gaseous inlet and outlet, a reactor vessel, a vacuum pump, and a matching box. Reactors vary in size from 1 liter to several cubic meters.

When the plasma contacts with the biomaterial surface, the activated species initiate chemical and physical reactions. As a result, alternation of surface properties and surface morphology occurs. This process of surface modification by plasma is called *plasma treatment.*[14] Likewise, when plasma interacts with organic molecules in vapor, polymers are formed, and whole surface of substrates in the plasma zone are coated with the polymers. This process of polymer formation by plasma is called *plasma polymerization.*[18]

23.2.2 Plasma Sputtering and Etching

Plasma sputtering is a simple plasma-surface treatment method. During the sputtering process, a negative voltage is applied to the substrate and plasma is generated by radio frequency glow discharge (RFGD). The ions are accelerated toward the substrate by the applied electric field. Some surface atoms will acquire enough energy and escape from the substrate into the vacuum chamber. With sufficient sputtering time, surface contamination can be cleaned off and degradation processes can begin. The etching can be used as a pretreatment for subsequent *implantation* and *deposition*. The interaction between the plasma and polymer leads to two competitive reactions – modification and degradation. When the modification effect dominates, the properties of the biomaterial will change due to the *ion beam interaction*. When degradation is prominent, etching will take place on the polymer surface. Two types of degradation reactions occur, namely, *chain scission* and *crosslinking*. In most polymers both of them have a place, but one is always predominant over the other. Polymers having a repeating unit of $–CH_2–CHR–$ undergo mainly chain scission and low-molecular-weight products are formed; those with $–CH_2–CHRR´–$ undergo crosslinking reactions and this is due to the resonance stability of formed radicals.[14,18] Crosslinking reactions consist in the formation of intermolecular bridges. The resulting layers act like a barrier and decrease the diffusion of any type of molecule.

Regarding the weight loss dependency from the nature of the modified materials, it should be noticed that those ones with oxygen containing groups are more sensitive.[19] Degradation yield and

rate are also dependent on plasma nature. O_2, He, and N_2 plasma sputtering was performed on poly(*L*-lactic acid) (PLA) and it was found that the resulted patterns are different.[20] Once again, it should be underlined that all those processes are restricted to the topmost layer and therefore the modified material has similar chemical and physical properties to the original one.

An example[21] for successful application of this method is the use of CH_4 plasma treatment for adhesion enhancing between the reinforced absorbable calcium phosphate fibers and the absorbable polyglycolide acid (PGA) matrix (calcium phosphate/PGA composites have been widely proposed for totally absorbable fracture plates). The observed improvement has been partially attributed to a better microscopic mechanical interlocking between the plasma-treated calcium phosphate fibers and the PGA matrix, but the treatment has also resulted in lower *surface energy.* Surface porosity of cellulose acetate membranes[22] has been tailored by CO_2 plasma etching. The structure of the top layer has been changed from nonporous to nanoporous, up to complete removal of the skin by accurately increasing the treatment time.

23.2.3 Plasma Functionalization

Plasma treatment can be used to create a functionalized surface through attachment of new chemical groups or atoms. In a typical plasma implantation process, hydrogen is first abstracted from the polymer chains to create radicals which then recombine with simple radicals created by the plasma gas to form hydrophilic or hydrophobic surfaces. The use of oxygen (–OH, –C=O, –COOH groups introducing) or nitrogen ($-NO_2$, $-NH_2$, $-CONH_2$ groups) plasma is one of the most powerful methods for increasing of material hydrophilicity, which usually resulted in improving of the adhesion strength, biocompatibility, and other pertinent properties.[19,23,24] This was confirmed by oxygen implantation in starch-based biomaterials. The implantation resulted in a simultaneous enhancement of cell adhesion and on induction of a bioactive behavior.[25] Polystyrene,[26] poly(methyl methacrylate),[27] and polyethylene therephthalate[28,29] are just few of the biomaterials that showed increased surface wettability and enhanced cell adhesion after oxygen plasma treatment. After studying a wide variety of substrate polymers, Tamada and Ikada[30] found that there is an optimal wettability for cell adhesion and that is approximately 70° water contact angle. Polyethylene, polytetrafluoroethylene, poly(ethyleneterephthalate), polystyrene, and polypropylene films have been the studied materials. A considerably high degree of functionalization with primary amino groups on polyvinylidenefluoride microfiltration membranes has been done by Muller et al.[31] using nitrogen implantation. Satisfactory results have been achieved by Si^+ and N^+ ion implantation in several polymer biomaterials such as silicone rubber and polyurethane. It has succeeded in improving wettability, anticoagulability, and anticalcific behavior of polyurethane and critical surface tension of silicon rubber, which is thought to be a primary cause for the biofouling.[32,33] Another successful application[34] of this method is the use of silver negative ions implantation for improving biocompatibility of polystyrene dishes. The modification has resulted in lower contact angle and cell attachment and growth of human umbilical vascular endothelial cells has been observed only for modified surfaces.

For some applications as intraocular lenses or hemodialysis membranes, hydrophobic surfaces are needed. Most hydrophobic surfaces of biomaterials are formed by CF_4 plasma. Low surface energy and therefore antiadhesion properties are some of the introduced surface properties that prevent biofilm formation.

In both cases, hydrophilic and hydrophobic surface alternation, the final materials properties are strongly dependent on used dose and energy of implantation.

23.2.4 Dual Plasma Deposition

Plasma deposition is a thin film technique. A layer with properties distinctly different from those of the bulk materials can be synthesized on their surfaces. In this process, gas and metal plasmas are simultaneously generated, usually by RFGD sources. The composition of the film can be

controlled by adjusting the flow rates of the gas. In this way, a film composed from several elements (gaseous and metallic) with various compositions can be fabricated in the same instrument without braking vacuum. Dual plasma deposition has been used to synthesize TiN and TiO_2 thin films using Ti cathode. Surface modification by titanium nitride film deposition has shown great potential for improving the hemocompatibility of biomedical materials and devices.[35–38] The efficiency of titanium oxide films for improving blood compatibility of heart valve materials has been also studied.[39–42] It has been found that it is strongly dependent on its thickness; the absorption ratio of albumin/fibrinogen has increased (a contribution to improve blood compatibility) with the increase of the thickness of deposited layer.[41]

23.2.5 Plasma Polymerization

Plasma enhanced chemical vapor deposition or more commonly called plasma polymerization employs a low pressure, flowing gas system in which ionized gas plasma is generated. Reactions among active species in the plasma lead to the formation of polymer deposited on the surface of a targeted substrate. As in the case of the plasma functionalization, plasma polymerization can yield hydrophobic or hydrophilic layers, depending on the monomer structure. Wide ranges of monomers are available for this process. In fact, virtually any organic molecule may be employed, providing it has adequate vapor pressure under vacuum conditions, in many cases leading to films with unique composition. It should be noticed that plasma polymerization is chemically different from conventional polymerization involving radicals and ions and polymers formed in this way have different chemical composition as well as different chemical and physical properties. Plasma polymers do not comprise repeating monomer units, but instead complicated units containing cross-linked, fragmented, and rearranged units from the monomers. In most cases, they have a higher modulus and do not exhibit a distinct glass transition temperature.

The possibilities which this technique offers have been used for increasing the biocompatibility and hemocompatibility of different materials membranes.[43] For instance, the deposition of thin fluorocarbon coating onto polysulfonate and poly(hydroxybutyrate) membranes has given very smooth and hydrophobic surfaces with reduced thrombogenicity and makes them useful for bioartificial pancreas devices. A plasma polymerized 2-hydoxyethyl methacrylate (HEMA) film[44] has been prepared by plasma polymerization onto silicon rubber for improving cell attachment and growth. A confluent cell layer has been observed after 72 hours for modified material compared with negligible cell attachment onto the control and the Ar plasma-treated surface. Plasma deposition of allylamine onto polyethyleneterephtalate (PET) membranes[45] has resulted in a surface carrying amine groups. It has been shown that the modified PET membranes with allylamine acquire tissue compatible properties, that fibroblasts adhesion and activity is enhanced in comparison to plain PET. Furthermore, the amine functionalities presented on the membrane surface could be also used for the covalent binding of ligands such as heparin by a simple wet chemistry to adapt one side of the membrane for the certain application, such as blood contact.

23.3 GRAFTING

Grafting has advantages over other methods in several points, including covalent attachment of graft chains onto a polymer surface avoid their delamination and ensure long-term stability of introduced chains, in contrast to physically coated polymer chains. The grafting methods can be generally divided into two classifications,[46] i.e., "grafting-from" and "grafting-to" processes. The former utilizes active species existing on the polymer surfaces to initiate the polymerization of monomers (usually acrylic or vinyl) from the surface toward the bulk phase. In the case of "grafting-to method," preformed polymer chains carrying reactive groups at the end or the side chains are covalently coupled to the surface. The "grafting-to method" will be dealt with in this article.

The fundamental step in grafting is the creation of reactive groups on the substrate surface. This could be done either chemically (Ce ions,[47–49] H_2O_2,[50–52] peroxide initiators,[53,54] etc.) or more often by irradiation (UV, ionizing irradiation, or glow discharge).[55] The great majority of grafting processes involves a radical mechanism of polymerization of vinyl monomers.

Plasma- and chemical-induced graft polymerization of acrylic monomers on starch-based biomaterials was performed[50] to improve cell adhesion and proliferation on the surface of the polymers, in order to adequate their properties for bone tissue engineering scaffold applications.[50] Radio frequency plasma (Ar/O_2) or immersion in a $H_2O_2/(NH_4)_2S_2O_8$ solution with UV radiation was used as the activator. The studied properties after grafting showed that the plasma-induced graft polymerization is more homogeneous in terms of surface modification than the chemical-induced treatment which also modified some extent the bulk of the material. Both surface activations resulted in grafted materials which are able to form CaP layers after incubation in a simulated body fluid (SBF).[50,52] The modified materials showed a remarkable improvement in cell adhesion/proliferation of goat bone marrow cells with respect to the original starch-based blends. To prevent the interaction between the blood and the material surface resulted in thrombus formation, Khorasani et al.[56] have grafted HEMA on silicone by laser-induced graft polymerization. Surface grafted poly(ethylene glycol) on different materials has been also used to prevent protein adsorption on the surface.[57] Polyolefins, which are widely used in the biomedical field due to their suitable bulk properties, have been functionalized using acrylic monomers and plasma activation.[53,58,59] The grafted materials have shown higher water wettability, which in most cases resulted in better cell adhesion.[5–7] The same behavior has been observed for polyurethane membranes grafted with acrylamide or itaconic acid monomers. The modified, more hydrophilic surfaces are useful for enzyme immobilization, weathering, blood compatibility, and less tissue damage for intraocular lenses.[47,55] Sometimes the grafted monomer can be used like a chemical handle for immobilization of other molecules. For example, polyacrylic acid has been introduced onto silicone rubber by plasma-induced graft polymerization and then linked with collagen to improve cell migration, attachment, and growth.[60] A series of surface coatings have been prepared by modifying the Ar plasma–treated polytetrafluoroethylene (PTFE, Teflon) and polyethyleneterephthalate (Dacron) grafts with collagen IV and lamini and subsequently bioactive molecules such as PGE_1, heparin, or phosphatidyl choline via the carbodiimide functionalities. The modification has aimed to find nonthrombogenic material having potency to be used for small diameter vascular graft applications.[12]

23.3.1 Chemical Treatments

Chemical surface modification can be performed either by direct chemical reaction with a given solution (wet treatment) or by covalent bonding of suitable macromolecular chains to the sample surface (grafting).[61–65]

23.4 WET CHEMISTRY

Wet treatments have been the first surface modification techniques used in order to improve surface properties of polymers. The chemical composition of the solution employed in the treatment has been mutated from general wet chemistry knowledge; for instance, hot chromic acid has been used to oxidize polyolefins. In other cases, however, specific solutions have been developed in order to exploit specific liquid–polymer interactions.

The comparison with solution organic chemistry is a key issue when discussing wet treatments of organic polymers. The basic question is, How is the reactivity of functional groups affected by the reduction of dimensionality, when the reaction occurs at an interface? Another important point involves where the reaction actually occurs. Depending on solvent–polymer interaction, one can expect a reaction confined to a nearly geometrical interface or extending far into the substrate.

The ultimate goal of this approach is to create well-defined functional substrates characterized by controlled surface properties or available for further chemistry.

The most common wet modification methods that have been used in industry to treat large objects that would be difficult to treat by other commonly used techniques such as flame and corona-discharge treatments are alkaline or acid etching,[66–68] oxidation (through ozone[69–71] or using other oxidizing agents[72–75]), and hydrolysis of polyester and other polymers.[76–79]

23.4.1 Etching and Oxidation

Chemical etchants are used to convert smooth hydrophobic surfaces to rough hydrophilic surfaces, usually by means of dissolution of amorphous regions and surface oxidation. Improved hydrophilicity resulting from surface modification has been found to be responsible for increasing cell adherence and proliferation in different materials and with several cell types.[80–82] Different oxidizing solutions could be used. The most common system[16] is a chromic acid solution — $K_2Cr_2O_7$, H_2O, and H_2SO_4 in different ratios. It has been used for oxidation of low-density polyethylene (LDPE), high-density polyethylene (HDPE), and polypropylene (PP). The results have shown that contact angles are lower for all of them, but PP is etched more readily than LDPE, which in turn is less resistant than HDPE. More of the biodegradable polymers are quite sensitive to very concentrated sulfuric acid, and this requires the use of oxidizing solution other than $K_2Cr_2O_7$–H_2SO_4. Carbon fiber-reinforced polyetherether ketone (PEEK) is presently being investigated[83] for manufacturing medical instruments, hip joint endoprostheses, and fracture fixation plates. Chemical etching with potassium permanganate has shown to enhance surface energy and wettability of PEEK as well as the surface roughness. The same oxidizing system was used for starch-based blends, which have been proposed for several biomedical applications, including bone fixation/replacement,[84,85] filling of bone defects, partially degradable bone cements,[86–88] drug delivery carriers,[89,90] and tissue engineering scaffolds.[91–93] For the latter application, it is of utmost importance to optimize cell adhesion and proliferation.

The modification by $KMnO_4/HNO_3$ resulted in a more hydrophilic surface with bigger polar component.[94] An explanation for this result is the formation of new oxygen-containing groups as it was observed by x-ray photoelectron spectroscopy (XPS) and Fourier transform infrared spectroscopy–attenuated total reflectance (FTIR-ATR). The performed tests with the modified materials showed simultaneously an enhanced cell adhesion and proliferation and a surface controlled bioactivity behavior (Figure 23.2 and Figure 23.3).

23.4.2 Hydrolysis

Two aspects of hydrolysis should be always considered. The first one is that all biomaterial surfaces are potentially susceptible to hydrolysis, simply due to the fact that they exist in a warm aqueous environment. As far as we are discussing degradable biopolymers, this process is most probably due to occur. Some classes of polymers containing ester, amide, or other carboxylic acid derivative groups undergo degradation by a simple hydrolytic mechanism (Figure 23.4). The reaction is base-catalyzed and sensitive to temperature above 37°C.

Typical examples are polymers deriving from polycondensation of lactic, glycolic, and hydoxybutyric acid. Degradation process for poly(ethylene oxide)/poly(ethyleneterephthalate) — a biodegradable copolymer used in surgery — has been studied[95] and confirmed this mechanism. The periodic increasing in –COOH content between 3 and 8 weeks has proved the continuing ester degradation and the solubilization of degradation products.

The second aspect is the hydrolysis utility like a surface modification method; the attack of a nucleophil agent could be used for increasing the number of hydrophilic groups and in this way to improve moisture-related properties or to use formatted groups in next reaction for immobilization or modification. The hydrolysis takes place in solvents and they should be chosen in order not to

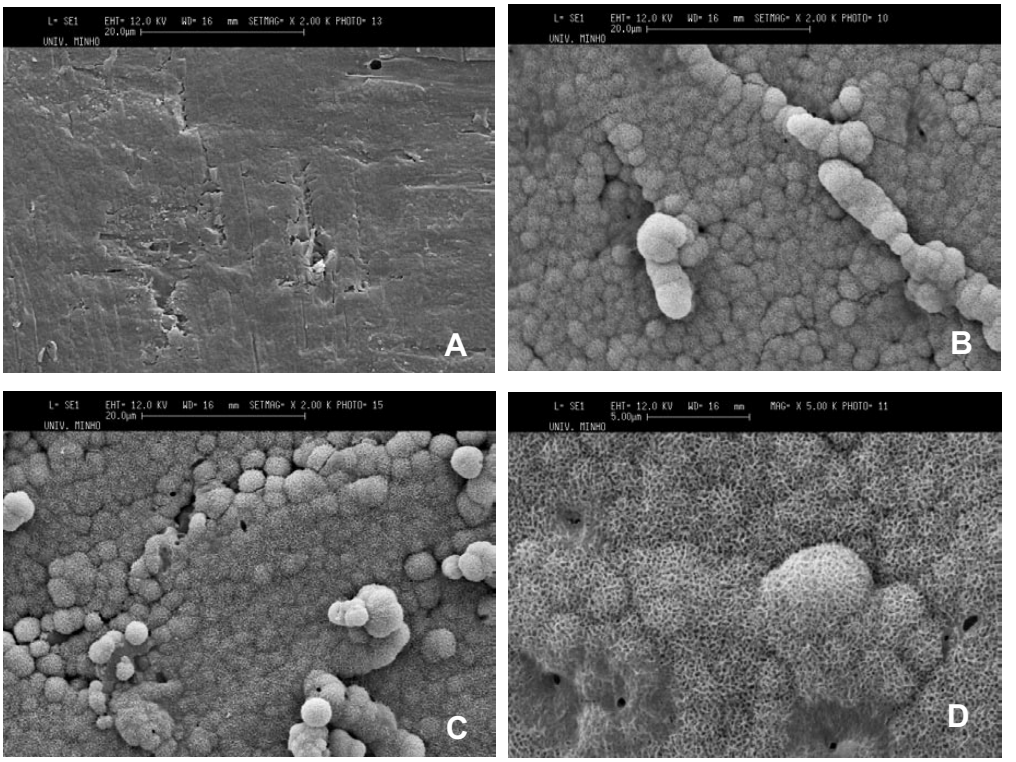

FIGURE 23.2 Starch/cellulose acetate blend (50/50) (SCA) modified with $KMnO_4/HNO_3$ and immersed in simulated body fluid for 1 (A), 3 (B, D), and 7 (C) days.

have any swelling action on the modified materials. Sodium and potassium hydroxides are most often used nucleophils. Surface functionalization of poly(ethyleneterephthalate) films has been performed using organic synthesis at the solid–liquid interface. Sodium hydroxide in acetonitrile has been used like a nucleophil in the first, hydrolyzing step of this modification. A surface with increased hydrophilicity and ability for adsorption or grafting of biochemical signals (extracellular matrix constituents, growth factors, or synthetic peptides) to promote cell cultivation has been the typically obtained result (hydroxyl chain end, Figure 23.5).[96,97] The same properties have been observed for hydrolysis products after $KMnO_4/H_2SO_4$ oxidation (carboxyl chain end, Figure 23.5).

Another sample for hydrolysis benefit is the chitosan preparation from chitin by simple hydrolysis with sodium or potassium hydroxide (Figure 23.6).

Chitosan membranes and fibers can be easily formed due to efficient inter- and intramolecular hydrogen bonding. Chitosan sponges and porous films are proposed as wound dressing materials, as physical barriers to prevent post-surgical adhesion, and as artificial skin. To improve blood compatibility, a surface modification with anionic modifiers including heparin, dextran sulfate, anionic phospholipids, and anionic derivatives of water-soluble polymers such as poly(ethylene glycol) (PEG) or poly(ethylene oxide) has been proposed.[98] These modified materials have shown lower thrombogenicity.

23.5 STERILIZATION

Clinically appropriate sterilization of any implantable material is necessary; anyhow, the mechanical properties as well as the key surface properties of the material should not be altered (unless they are in any way improved in a controlled manner). A variety of studies has shown that an unsuitable

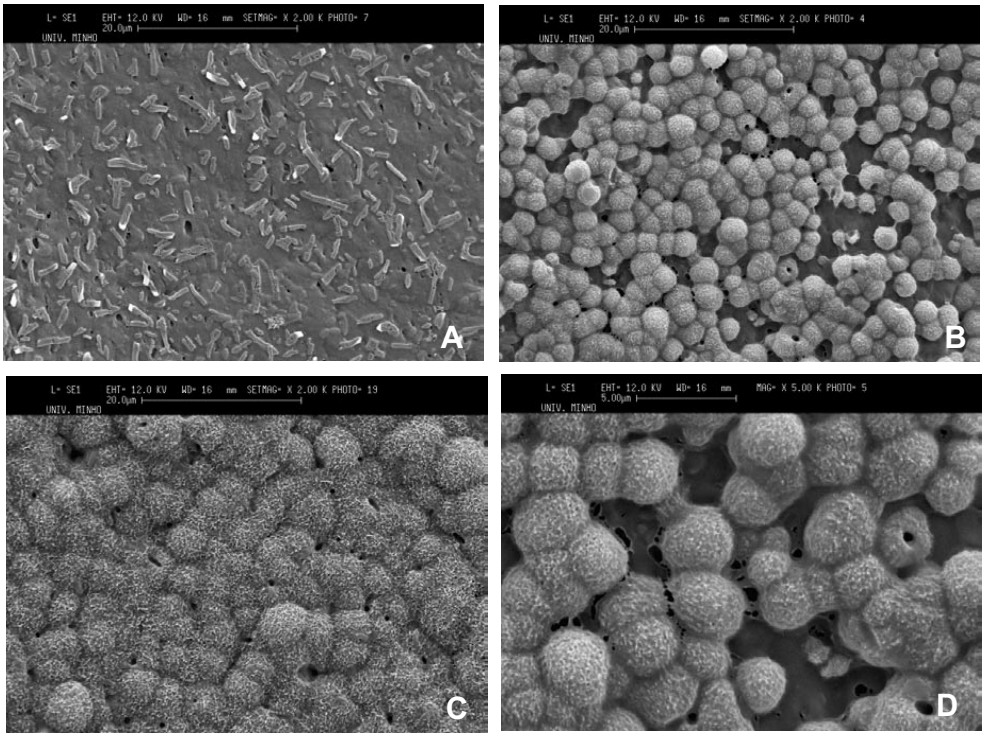

FIGURE 23.3 Starch/polycaprolactone blend (30/70) (SPCL) modified with $KMnO_4/HNO_3$ and immersed in simulated body fluid for 1 (A), 3 (B, D), and 7 (C) days.

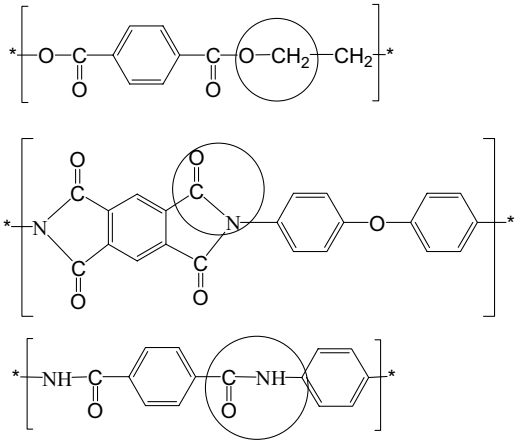

FIGURE 23.4 Some polymers and bonds which are suitable for modification by hydrolysis.

sterilization process can destroy the structure of the material, break chemical bonds, and alter physical, chemical, and biological properties substantially. The surface is not an exception; surface chemistry and surface energy could be inadvertently altered by cleaning and sterilization procedures. The perfect sterilization procedure for degradable biomaterials will be the one that does not include changes in the chemistry, the mechanical properties, and the degradation behavior. Routinely used sterilization processes for medical products[99–106] are steam, dry heat, ethylene oxide gas (EtO), and γ-irradiation. Among these, high-pressure steam (autoclaving) and dry heat are carried out at high temperature and can cause severe degradation and hydrolysis. In this respect, EtO and γ-irradiation

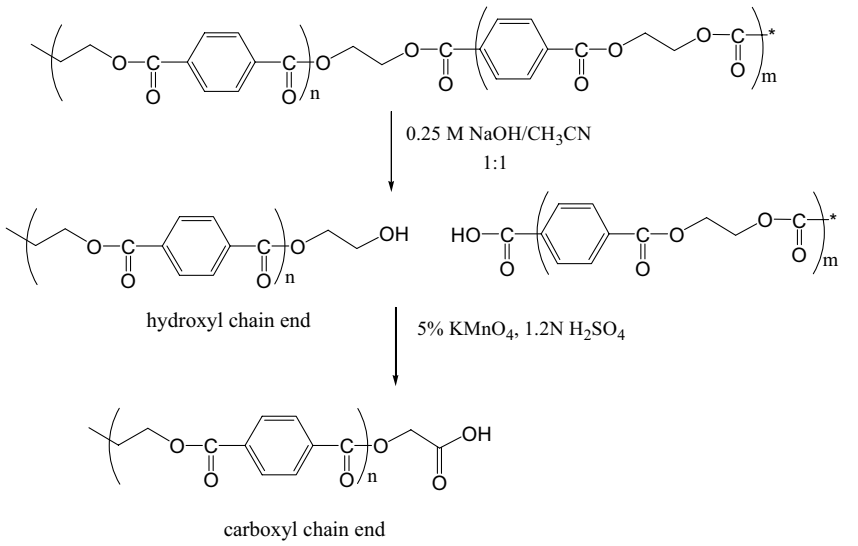

FIGURE 23.5 Naturally occurring or chemically created chain ends in poly(ethyleneterephthalate) film and membrane.

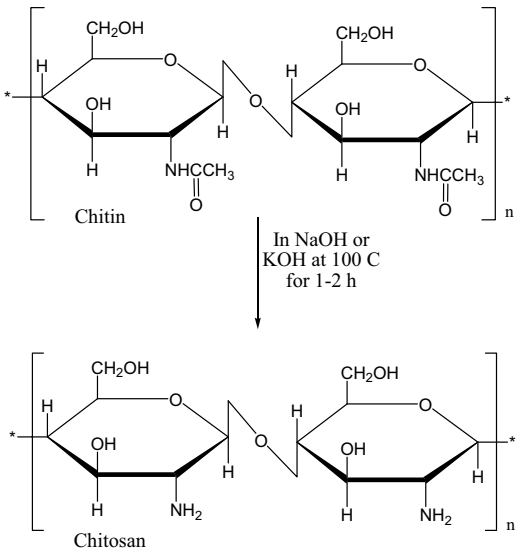

FIGURE 23.6 The chemical reaction involved in the conversation of chitin into chitosan.

techniques are preferred for the sterilization of biodegradable polymers.[107, 108] Glow discharge or gas plasma treatment, using plasmas of air, oxygen, nitrogen, hydrogen peroxide, helium, or argon, has been also shown to clean some implant surfaces.[109–114] All those methods have positive and negative sides regarding surface properties and chemistry.

23.5.1 γ-Irradiation

Radiation with γ-rays at doses exceeding 15 kGy is often used to sterilize extracorporeal and intracorporeal medical devices made from polymers. High-energy radiation, in addition to killing bacterial life, may also affect material properties. The primary changes can be chain scission or crosslinking. Material degradation leads to a loss of mechanical properties as well as to change in

the surface roughness, while crosslinking results in both an initial tensile strength increase and surface hydrophilicity decrease and in the properties related with it. In general, aromatic polymers are more resistant to high-energy radiation than aliphatic polymers, while the presence of impurities and additives may enhance degradation or crosslinking.[115]

Studies[107,116] on the effect of gamma irradiation on collagen structure clearly indicate chain scission resulting in a fraction of lower-molecular-weight material. Affato et al.[117–119] have studied the effect of sterilization method on the wear of ultra-high-molecular-weight polyethylene (UHMWPE). They have reported that EtO sterilized cups wear at a higher rate than those γ-sterilized. The higher crystallinity has been observed for γ-sterilized materials; chain scission is known to result in reduction of molecular weight and in corresponding increase in density and crystallinity since shorter chains can easily organize into more ordered arrangements. The same effect, chain scission, has been also observed[115] for γ-irradiated biodegradable polyurethanes in contrast to biostable polyurethanes. The scission has proceeded via the ester and urethane bonds, while the ether bonds have been not affected. Despite the decreasing in the polymer molecular weight and hence accelerating degradation over time, γ-sterilization has been found to be efficient sterilizing and preserving the morphology of a three-dimensional poly(lactide-co-glycolide) scaffolds in contrast with some other sterilization techniques.[120]

23.5.2 Ethylene Oxide Sterilization

EtO sterilization has been exploited as a low-temperature process. That is compatible with a wide range of devices, has high penetration ability, and it is quite effective. The main disadvantage centers on EtO residuals with respect to both the device and release into the environment. Besides this, some chemical and physicochemical properties were found to be changed after the sterilization process.

Ethylene oxide is three member cyclic ether and the angle between the bonds is 60° instead the ideal one of 109°. This ring strain make it unstable and it reacts in nucleophilic substitution without acids or Lewis acids via cycle opening. Epoxides react cleanly with amines to give amino-alcohols. Surfaces or materials which have presented these groups will be chemically altered after sterilization. A typical example for such modification after sterilization is the collagen.[116]

EtO treatment is used to sterilize collagen reliably. This procedure requires humidification of the sterilization chamber and slightly elevated temperature. Under these conditions, only a little denaturation occurs. However, amino acid analysis indicates intensive reaction of ethylene oxide with the amino groups presented by collagen in the form of lysine and hydroxylysine residues and leads to an increase in pH.[116,121] Changes in the chemical and morphological properties for biodegradable implants from poly(trimethylene carbonate)/poly(adipic anhydride) blends have been also observed after EtO sterilization.[122] Despite its relative chemical inertness, EtO sterilized orthopedic UHMWPE has presented surface oxidation. The oxidized species have been esters and acids, which according to the performed analyses are not related with chemical reaction with ethylene oxide but originating from the decomposition of primary peroxides, which can be formed during the shaping of the polyethylenes.[123] The oxidation level of EtO sterilized polyethylenes is at least one order of magnitude smaller than that of γ-irradiated materials, which makes the former method more appropriate. On clinical experiences, EtO gas sterilized UHMWPE components showed favorable consensus. Comparative studies on γ-sterilized and EtO sterilized components demonstrated that the latter type showed significantly less surface damage and delamination than the former one.

23.6 CHARACTERIZATION

The traditional methods used to analyze the bulk structure of the materials are not suitable for surface determination. It should be noticed that all possible methods that are used to analyze the surfaces have the potential to alter the surface.[13] Moreover, because of the potential for artifacts

TABLE 23.2
Some Methods for Surface Analysis and Their Characteristics

Method	Depth Analyzed	Spatial Resolution	Analytical Sensitivity
Contact angles	320 Å	1 mm	Low or high depending on the chemistry
XPS	10–250 Å	10–150 μm	0.1 atom%
FTIR-ATR	1–5 μm	10 μm	1 mol%
SEM	5 Å	40 Å	High but not quantitative

and the need for many pieces of information to construct a complete picture of the surface, more than one method should be used whenever possible. The basic used techniques as well as their sensitivity are given in Table 23.2.

23.6.1 Contact Angle Measurements

Surface atoms and molecules are in a different environment compared with their bulk counterparts; they are subjected to intermolecular attraction from one side only. The energy of the surface, which is directly related to its wettability, is a useful parameter that has often correlated strongly with biological interaction. Unfortunately, there are no direct methods to measure surface energy or surface tension of solids. However, a number of indirect empirical and semiempirical methods have been developed based on contact angle measurements.[13,124]

The basic relationship describing the force balance at a three-phase boundary (Figure 23.7) is

$$\gamma_{lv}\cos\theta = \gamma_{sv} - \gamma_{sl} \tag{23.1}$$

where γ_{lv} is liquid–vapor surface tension, γ_{sv} is solid–vapor surface tension, and γ_{sl} is solid–liquid surface tension.

Various approaches have been developed to calculate surface tensions of solids by contact angle measurements. The most-used methods are critical surface tension by Zisman,[125–128] the equation of state of Wu[129–30] and Neumann,[131] Fowkes theory,[132,133] the extended Fowkes equation, and acid-base interaction.[134–136]

Kaeble's equation[137,138] is frequently used to determine the polar components of polymer surface tension by measuring the contact angles of two liquids of known surface tension on polymer surface:

$$\gamma_{lv1}(1 + \cos\alpha_1) = 2[(\gamma^d_{lv1} + \gamma^d_{sv})^{1/2} + (\gamma^p_{lv1} + \gamma^p_{sv})^{1/2}] \tag{23.2}$$

$$\gamma_{lv2}(1 + \cos\theta_2) = 2[(\gamma^d_{lv2} + \gamma^d_{sv})^{1/2} + (\gamma^p_{lv2} + \gamma^p_{sv})^{1/2}] \tag{23.3}$$

where subscripts 1 and 2 are for liquids 1 and 2, respectively. The increase in polar component has been used as an indicator for the increase in the polar groups on a treated polymer surface. For example, γ^p_{sv}, γ^d_{sv}, and γ_{sv} were measured[50] for starch-based blends before and after grafting with

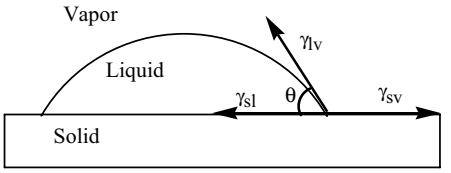

FIGURE 23.7 Equilibrium contact angle.

TABLE 23.3
Dispersive (γ^d_{sv}) and Polar (γ^p_{sv}) Components and Surface Energy (γ_{sv}) Values for Starch-Based Blends SEVA-C and SCA, Grafted with Acrylic Acid after Chemical or Plasma Activation

Material	γ_{sv}	γ^d_{sv}	γ^p_{sv}
SEVA-C	45.1	32.3	12.8
SEVA-C grafted with AA, chem. act.	51.7	37.5	14.2
SEVA-C grafted with AA, plasma act.	54.0	28.7	25.3
SCA	58.3	37.9	20.4
SCA grafted with AA, chem. act.	61.4	34.7	26.7
SCA grafted with AA, plasma act.	54.1	28.3	25.3

acrylic acid (Table 23.3). In all cases, after grafting, an increase in the polar component as well as in the surface tension was observed because of the introduced -COOH groups.

23.6.2 X-Ray Photoelectron Spectroscopy (XPS)

Although some polymers such as fluoropolymers and poly(vinyl chloride) have been shown to suffer x-ray damage after prolonged exposure, the x-ray photoelectron spectroscopy is a commonly used method for surface chemical analysis. During the XPS experiment, a sample is exposed to x-ray irradiation; x-rays interact with substrate surface atoms, and this interaction causes an emission of a core level electron. The value of this electron energy provides information about the nature and the environment of the atom which has owned it. Binding energies of some atoms are presented in Table 23.4.

The exact core level binding energy for any given atom varies slightly depending on its local bonding situation, producing so-called binding energy or "chemical" shifts (Table 23.5).

Unfortunately, the range of these shifts is small and when the polymer structure involves the same atom in more than one chemical environment, the resulting chemically shifted peaks overlap and produce a complex end signal. This problem is greatest of course for C1s core level spectrum.[139]

This already referred to starch-based blends, which were also analyzed by XPS. The spectra (Figure 23.8) seem very similar for three of them; oxygen and carbon peaks are presented.

The differences in chemical composition caused from second components presented in the blends are obvious when we compare the components of carbon peaks (Figure 23.9). In SEVA-C spectrum (Figure 23.9A), only the characteristic for the carbon skeleton (C-C) peak and for

TABLE 23.4
Binding Energies of Most Common Elements for Degradable Biopolymers

	Binding Energies (eV)	
Element	1 $S_{1/2}$	2 $S_{1/2}$
H	14	
C	287	
N	402	
O	531	24

TABLE 23.5
Primary C1s Chemical Shifts (eV) for Oxygen Functions, Relative to Saturated Hydrocarbon (C1s = 285.00 eV)

Functional Group	Chemical Shift (mean)^{eV}	Functional Group	Binding Energy (mean) eV
C-O-C	1.45	C-**O**-C	532.64 aliphatic, 533.25 aromatic
C-OH	1.55	C-**O**H	532.89 aliphatic, 533.64 aromatic
C-O-C=O	1.64	—	—
C=O	2.90	C=**O**	532.33 aliphatic, 531.25 aromatic
O-**C**-O	2.93	**O**-C-**O**	533.15
O-C-**C**=O	3.99	C-C(O)=**O**	532.21 aliphatic, 531.65 aromatic
-**C**OOH	4.26	C-C(**O**)=O	533.59 aliphatic, 533.14 aromatic
O=**C**-O-**C**=O	4.41	O=C-**O**-C=O	533.91
		O=C-O-C=**O**	532.64
O-**C**(O)=O	5.40	**O**-C(**O**)=O	533.93
		O-C(O)=**O**	532.38

hydroxyl-bonded carbon (C-O) appear. New signal corresponding to -COOR group appears in SPCL spectra (Figure 23.9B) because of the polycaprolactone and in SCA (Figure 23.9C) because of the cellulose acetate.

23.6.3 Fourier Transform Infrared Spectroscopy — Attenuated Total Reflectance (FTIR-ATR)

FTIR-ATR spectrometry can provide valuable information related to the chemical structure of biomaterials. The spectra are obtained by pressing small pieces of material against an internal reflection element (IRE). IR radiation is focused onto the end of the IRE. Light enters in the IRE and reflects down the length of the crystal. At each internal reflection, the IR radiation actually penetrates a short distance (~ 1–5 μm) from the surface of the IRE into the polymer membrane. It is this unique physical phenomenon that enables one to obtain infrared spectra of samples placed in contact with the IRE.

Infrared spectroscopy provides information related to the presence or absence of specific functional groups (because of absorption of radiation which is related to vibrations of the chemical bonds), as well as the chemical structure of polymer materials. Shifts in the frequency of absorption bands and changes in relative band intensities indicate changes in the chemical structure or changes in the environment around the sample. Therefore, FTIR-ATR spectrometry can be used to determine the resulted surface chemistry after special chemical or physical treatments are applied. The chemistry of a viable biological fouling layer can also be studied.

An illustration that tries to show how informative could be FTIR-ATR is the spectra presented in Figure 23.10 and Figure 23.11.

The spectra for starch and its blends are completely different. New peaks in the blends spectra are characteristic for the second components in the blends. Very strong bands at about 1700 cm^{-1} characteristic for carbonyl groups appear in starch/cellulose acetate blend (-$COOCH_3$ groups) and starch/poly(ε-caprolactone) (-O-C=O groups) spectra. For starch/poly(ethylenevinyl alcohol), the second component has –OH characteristic band, which is already presented in starch spectrum and because of that both spectra are quite similar.

In Figure 23.11 spectra of starch/cellulose acetate blend before and after treatment are presented. The carbonyl band (at about 1700 cm^{-1}) disappears and the intensity of hydroxyl band (3000–3500 cm^{-1}) increases after treatment with $NaIO_4/NaBH_4$.

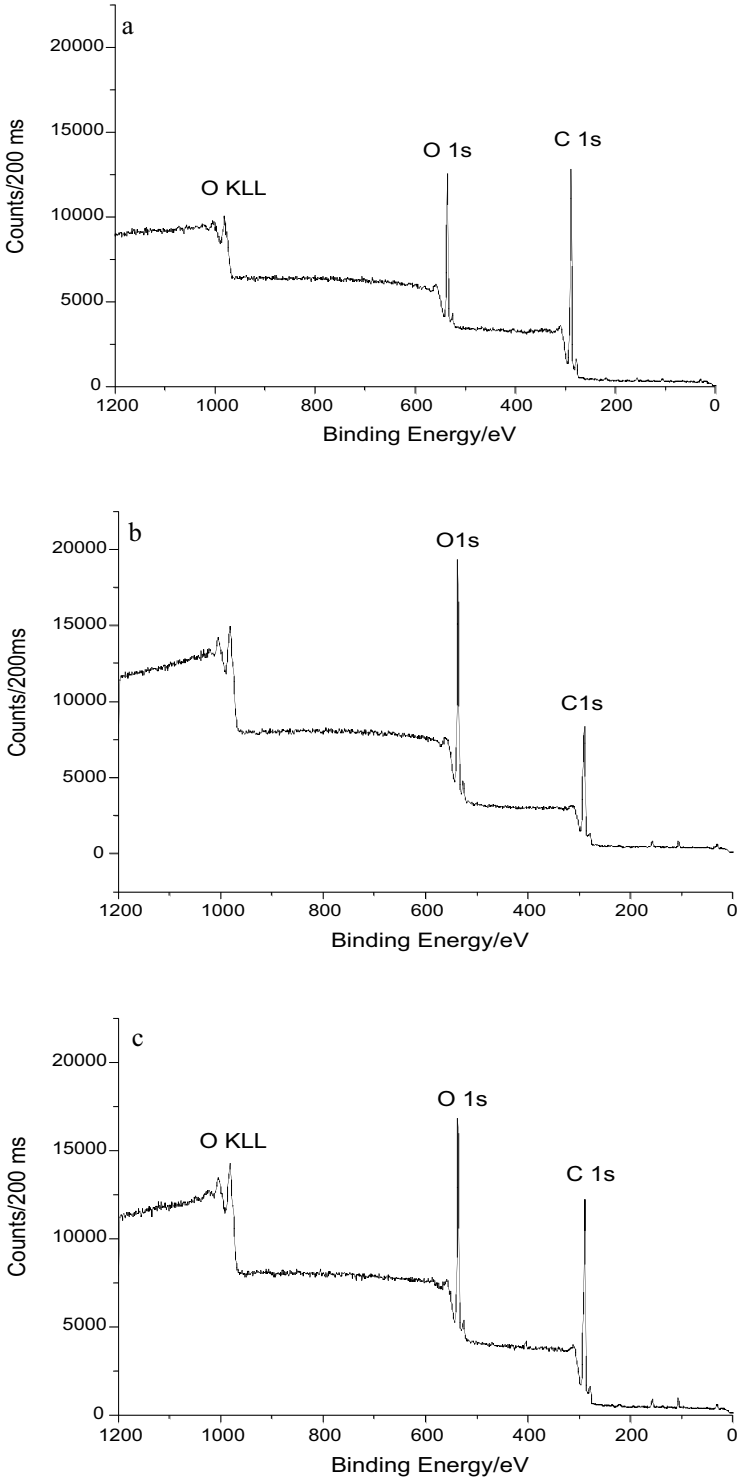

FIGURE 23.8 XPS analyses for SEVA-C (a), SCA (b), and SPCL (c) samples.

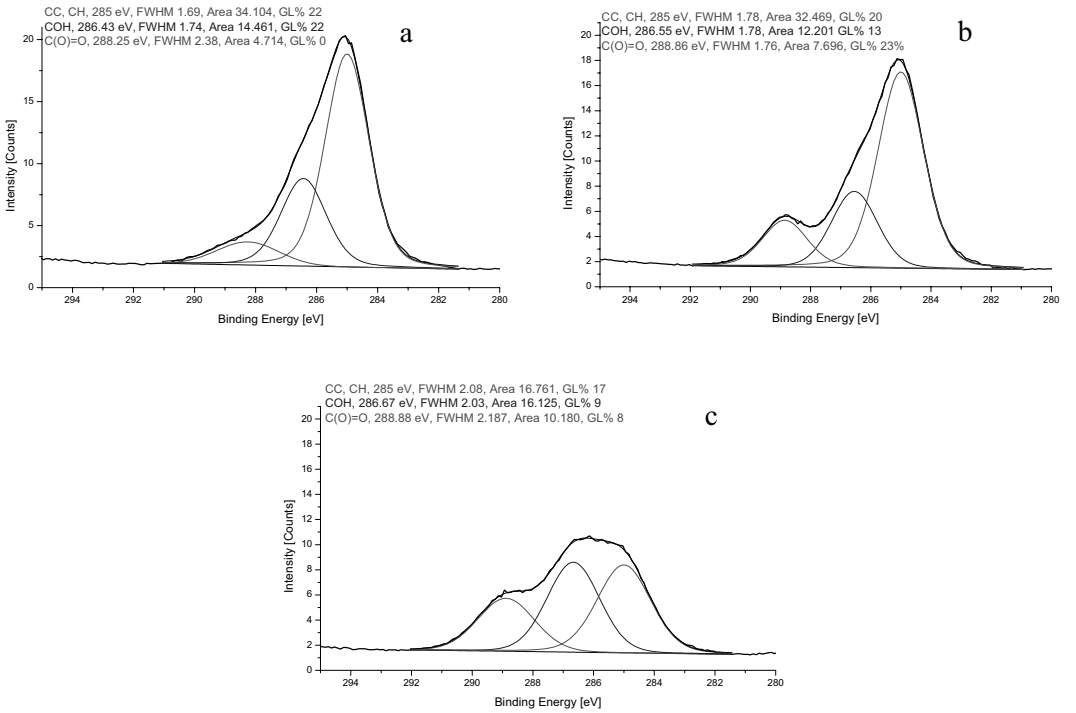

FIGURE 23.9 C 1s core level spectra of SEVA-C (a), SPCL (b), and SCA (c).

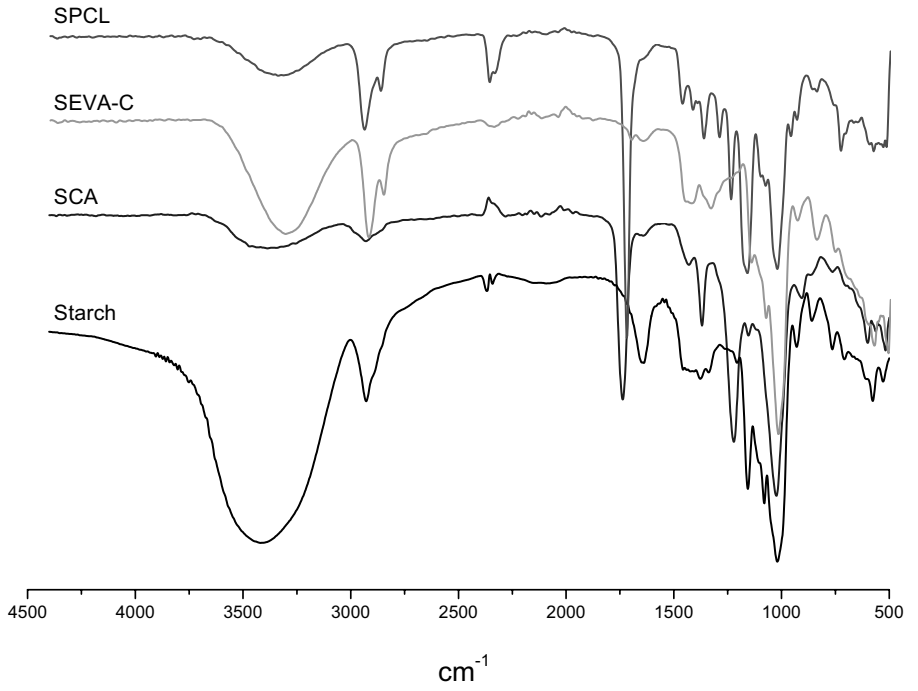

FIGURE 23.10 FTIR-ATR spectra of starch and its blends with cellulose acetate (50/50) (SCA), polyvinyl alcohol (50/50) (SEVA-C), and polycaprolactone (70/30) (SPCL).

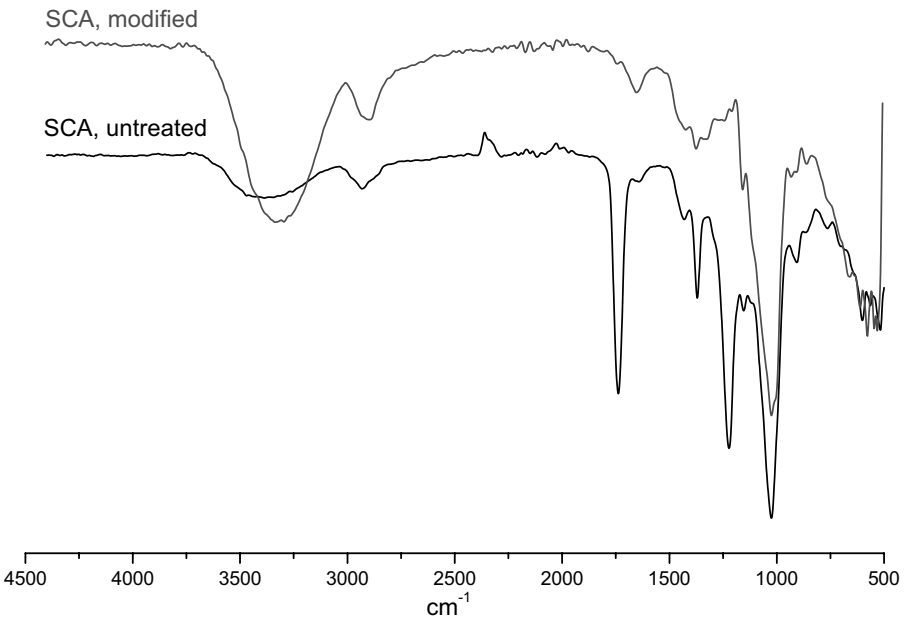

FIGURE 23.11 FTIR-ATR spectra of starch/cellulose acetate blend surface modified using $NaIO_4/NaBH_4$ system and the original one.

This is a confirmation for the performed reduction; all carbonyl groups obtained after $NaIO_4$ oxidation together with the ester groups from cellulose acetate are transformed in hydroxyl groups in the second reaction step.

23.6.4 Scanning Electron Microscopy (SEM)

The SEM uses a beam of electrons to scan the surface of a sample and to build very detailed three-dimensional image of the specimen. When the electron beam hits the sample, the interaction of the beam electrons from the filament and the sample atoms generates a variety of signals. Unlike the light microscope, in which light forms an instant "real image" of the specimen, the electrons in an SEM do not form a real image. Instead, the SEM scans its electron beam line by line over the sample (it is very similar to scan dark room from side to side using a flashlight or to the image building on a TV monitor).

Usually SEM is used together with other surface analysis techniques and helps in the interpretation of data received by them. The use of different magnification allows us to have very detailed pictures for the roughness and texture of material (Figure 23.12) and to see how a surface modification affects them (Figure 23.13).

23.6.5 Atomic Force Microscopy (AFM)

AFM images show three-dimensional information about surface features. The AFM can examine any rigid surface, either in air or with the specimen immersed in a liquid. Minor differences between relatively smooth surfaces can be resolved, even single atoms can be observed. However, AFM can also examine a field of view larger than 125 microns. The AFM works in much the same way as a phonograph needle scans a record. The tip is positioned at the end of a cantilever beam shaped much like a diving board. As the tip is repelled by or attracted to the surface, the cantilever beam deflects. The magnitude of the deflection is captured by a laser that reflects at an oblique angle from the very end of the cantilever. A plot of the laser deflection versus tip position on the sample

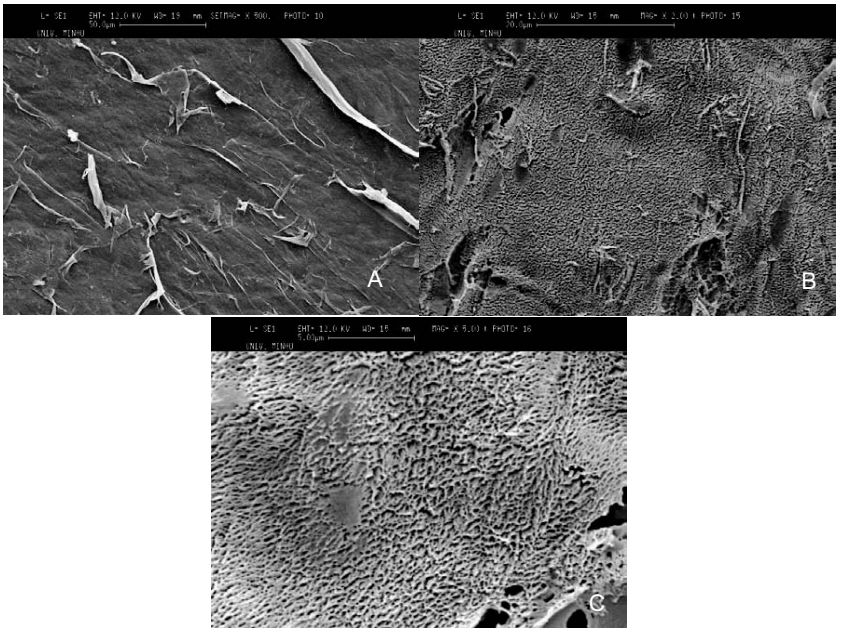

FIGURE 23.12 SEM pictures of SCA surface using different magnifications — 500× (A), 2000× (B), and 5000× (C).

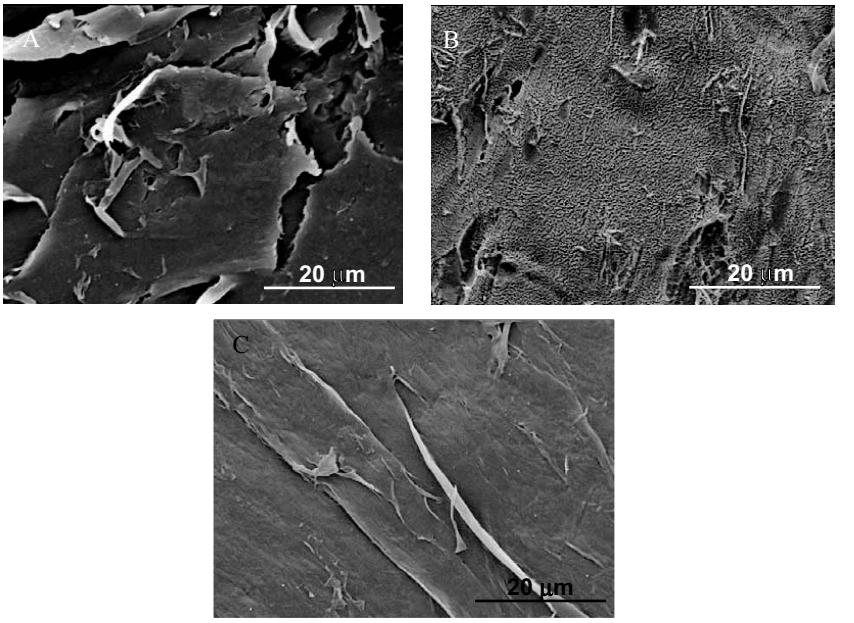

FIGURE 23.13 SEM pictures of untreated SEVA-C (A) and different modified (B and C).

surface provides the resolution of the hills and valleys that constitute the topography of the surface. The recent improvements in observation and measurement capabilities of this technique include the possibility of scanning the surface under aqueous conditions. Using this advantage, Leonor et al.[140] have investigated *in situ*, the *in vitro* bioactivity of partially crystallized 45S5 Bioglass® as a function of immersing time in simulated body fluid. Another main application of AFM is for

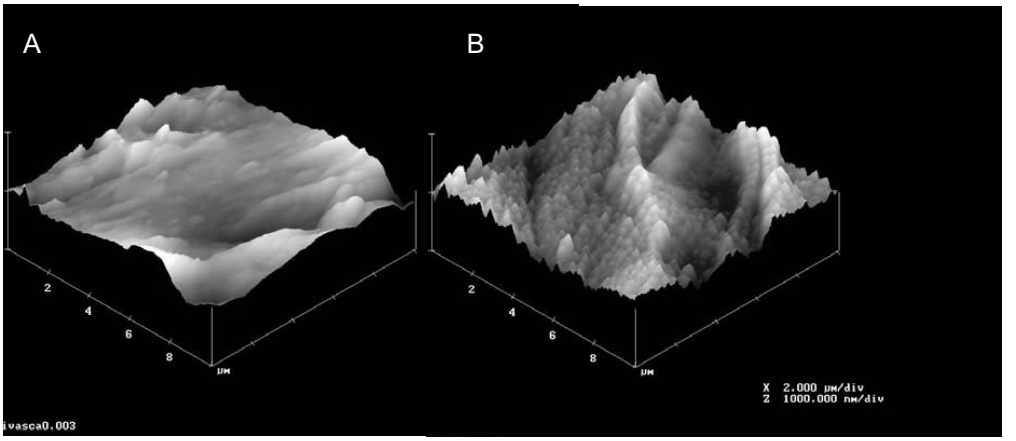

FIGURE 23.14 AFM images of starch/cellulose acetate blend before and after surface modification by Ar/O_2 plasma.

roughness analysis, since cell adhesion is dependent on surface roughness. Some AFM images are presented in Figure 23.14.

23.7 CONCLUSIONS

Biodegradable polymers have been shown to play an important role in a variety of medical applications. The need for a clearly biocompatible behavior will make surface treatment techniques more common in biomaterials science. These processes selectively modify the surface energy, enhance the bonding strength of substrates, minimize biofouling, etc. The most important advantage of those techniques is to change the surface properties to become more biocompatible or better mimic the local tissue environment without altering the bulk attributes, thereby offering a high degree of quality control, yield, reliability, and reproducibility.[1]

Until one knows precisely what result one wants (i.e., how to trigger a specific biological reaction needed to achieve a specific function), one still cannot know what surface structure must be created. Therefore, future research must be directed toward more fully understanding the mechanism and the effects of the interaction between polymers and biological systems. The understanding of this mechanism will help to manufacture improved polymeric systems specifically targeting a certain application.[141] Efforts are under way to develop surfaces that are specifically recognized by components in the biological environment. Strategies involve multifunctional surfaces to mimic receptor sites, template materials, and immobilized biomolecules to interact specifically with biological systems, among many others.

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Part V

Biodegradable Polymers for the Engineering and Regeneration of Different Tissues

24 Bone and Articular Cartilage Tissue Engineering: The Biological Components

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CONTENTS

24.1 INTRODUCTION

Bone and articular cartilage are two essential tissues within the human body.

Bone is a dynamic, highly vascularized tissue with a unique capacity to heal and remodel without leaving a scar.[1] These properties, together with its capacity to rapidly mobilize mineral stores on metabolic demand, make it the ultimate smart material. Its main role is to provide structural support for the body. Furthermore, the skeleton also serves as a mineral reservoir, supports muscular contraction, withstands load bearing, and protects internal organs.[1,2] On its side, articular cartilage is essential to normal diarthroidal joint, because of its ability to reduce joint stress and surface friction.[3] It is then logical to say that major alterations in their structure due to injury or disease can dramatically alter one's quality of life.

Although major progresses were done in the field of bone/cartilage regenerative medicine during the years, current therapies, such as bone grafts, still have several limitations, as it will be later discussed.

It is in this context that an emerging field of science called tissue engineering has been gaining notoriety in the last 10 years. As it was defined by Langer and Vacanti,[4] tissue engineering is "an interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function." It combines knowledge from physics, chemistry, engineering, materials science, biology, and medicine in an integrated manner.[4–6]

In order to fully develop a tissue equivalent, there are five aspects that should be taken care of[4–6]: (1) in order to grow in a 3-D manner similar to that found *in vivo,* cells will need a 3-D support, a scaffold that besides being a substrate for cell culture, will also confer mechanical stability to the construct until the neo-tissue is formed (subject that is not going to be discussed in the present chapter); (2) an adequate cell population to initiate the regenerative process; (3) growth and differentiation factors; (4) adequate cell culture methodologies; and (5) accurate animal models that mimic the conditions that are pretended to regenerate *in vivo*. The present chapter is focused on the four last points, that is, on the biological components.

24.2 CLINICAL NEEDS

24.2.1 Bone

As it was previously referred, bone is a remarkable tissue and plays a series of important roles in the daily life of our organism. Severe injuries to this tissue can lead to drastic changes in a patient's life quality. For instance, it can limit the capability of a person to walk or, in other cases, be the origin of serious social and psychological problems, when injuries are made at the craniofacial level.

Current methodologies used for bone replacement/regeneration are based on autologous and autogenous bone grafts or as an alternative to these, the use of metal and ceramics implants.[7–10]

Autologous bone graft (bone taken from another part of the patient's own body) and allogenous bone graft (bone taken from somebody else's body) have been one of the chosen methodologies of bone replacement for many years**.** In the first case, the grafts can be obtained from both cortical and trabecular bone. Usually the patient's iliac crest is commonly used as its source.[10] However, its application is limited, namely due to the limited amount of the autograft that can be obtained and donor site morbidity.[7–11] To overcome these negative aspects, allografts were put forward as an alternative. However, the rate of graft incorporation is lower than with the autograft. Furthermore, it could introduce the possibility of pathogen transmission from donor to host. Other negative aspects of this methodology are the infections that could occur in the recipient's body due to the transplantation and the possibility of rejection.[7–12]

Other routes used for bone replacement/regeneration have focused on the utilization of metals or ceramics. Nevertheless, these two options present some problems that do not allow them to be

the ideal materials for the referred applications. Metals, for instance, exhibit poor overall integration with tissue at the implantation site and fail because of infection or secondary to fatigue loading.[11] Furthermore, they are also too stiff, inducing in most cases "stress-shielding." On the other side, ceramics have very low tensile strength and are brittle and so they cannot be used in locations of significant torsion, bending, or shear stress and are not easy to manipulate by the surgeons.[11]

24.2.2 Articular Cartilage

Unlike other tissues, in which small defects are rapidly repaired without future problems, in cartilage even a small lesion can pose a major problem. In fact, this small lesion will not only fail to heal, but will almost certainly enlarge with time.[13] This is in part due to the tissue native properties. For instance, the chondrocyte to matrix ratio, mitotic activity, and turnover rate are very low, which by itself leads to a very low spontaneous repair.[3,14]

Articular cartilage defects can be divided in partial thickness defects and full-thickness defects.[3,13,14,15]

Partial thickness defects lie entirely within the confines of cartilage itself and do not penetrate subchondral bone. Because of this, blood-borne cells, macrophages, and mesenchymal stem cells are not able to migrate to the injury site.[3,13,15] Furthermore, because cartilage is an avascularized tissue, there is no blood, and hence no fibrin clot formation. In this sense, the lesion will not heal, remaining in an inert state. These kinds of lesions are commonly found in early osteoarthritic degenerative processes.[13]

Full-thickness defects span across the entire articular cartilage and can additionally penetrate the subchondral bone, being accessible to blood cells, macrophages, and MSCs. Due the natural wound-healing process, a fibrin clot will be formed in the injury site. This fibrin clot will act as a temporary matrix for MSC migration, conduction, and differentiation.[14] Although full-thickness defects commonly become filled with a tissue that resembles cartilage, this will not persist.[13] In fact, it will undergo a degenerative process after 6 to 12 months. Reasons by which the natural reparative fails are related with the lack of integration between repaired and native cartilage, mainly because collagen fibrils do not intermingle with one another, which at the same time can be related with the nature of cartilage extracellular matrix (ECM).[13]

Current treatments for the above referred to problems are based on conventional chirurgic methodologies and autologous/allogenous transplants/grafts. The first will not be discussed in the present chapter. For further information in this topic, the reader can consult the review by Hunziker.[16] For the second option, periostal grafts and chondral and osteochondral transplantation are the typically chosen methodologies.

The principle that lies behind the grafting of periosteum in cartilage injury sites is the observation that the cambial layer of this tissue possesses chondrogenic potential.[13–19] Nevertheless, and in spite of the fact of the existence of some clinical products based on this methodology, complete restoration of hyaline cartilage tissue or long-term stability of the neotissue is yet to be achieved. Poor fixation to the defect floor and uncontrolled calcification are the main problems when using this technique.[13]

In the case of autologous chondral/osteochondral transfer, also called mosaicoplasty, osteochondral cylinders are harvested from a joint area of minor load and press-fitted into predrilled holes at the defect site.[15] However, due to the limited availability of donor tissue and the induced morbidity at the donor site, the technique cannot be applied when the damaged area is above 2 cm^2.[15] Furthermore, animal studies have shown that on the long-range, mosaicoplasty was found to be associated with the rapid degeneration from both the transplanted cartilaginous tissue and the vicinal native chondrocytes.[16]

Allogenous chondral/osteochondral grafts could be an option to the autogenous one and would not have the donor morbidity site problem. However, problems such as the scarcity of fresh material, risk of disease transmission, few sources for biological material extraction, and inability

to adequately secure the graft (these last two mainly for chondral grafts) have refrained its use in clinical applications.[16]

24.3 BONE AND CARTILAGE STRUCTURAL BIOLOGY

24.3.1 Bone Biology

24.3.1.1 Cortical versus Trabecular Bone

Bone tissue in the adult skeleton is arranged in two architectural forms, trabecular, also called cancellous or spongy bone (around 20% of the total skeleton), and cortical or compact bone (around 80% of the total skeleton).[20–22]

The proportions of these two architectural forms differ at various locations in the skeleton. *Cortical bone* is almost solid. It is only 10% porous[20] and can be divided into different subgroups[20,22]: long bones (femur and tibia), short bones (wrist and ankle), and flat bones (skull vault and irregular bones). It is covered by a continuous outer layer sheath of connective tissue, called the periosteum.[22] *Trabecular bone* presents a higher porosity, 50–90%.[20] It is arranged in a sponge-like form, with a honeycomb of branching bars, plates, and rods of various sizes called trabeculae, and is commonly found in methaphysis of long bones, covered by cortical bone, and in the vertebral bodies.[20–22]

24.3.1.2 Woven versus Lamellar Bone

There are two types of bone that can be found within the skeleton: woven and lamellar bone.

Woven bone (Figure 24.1) is characterized by the random orientation of the collagen fibers, the osteocytes are large and extremely numerous and the calcification is delayed and does not proceed in an orderly fashion. Blood vessels are usually incorporated between the woven bone trabeculae, and will form the hematopoietic bone marrow.[22] For the reader's information, Figure 24.1 shows the typical organization of woven bone.

Lamellar bone (Figure 24.2) is generated more slowly than woven bone, presenting thicker collagen fibers with preferred orientation, alternating between layers or lamellae.[20] The main structure of lamellar bone is the osteon,[23,24] which is mainly composed of concentric collagen fibers, which are deposited around a central canal (Haversian Canal) containing a blood vessel, and

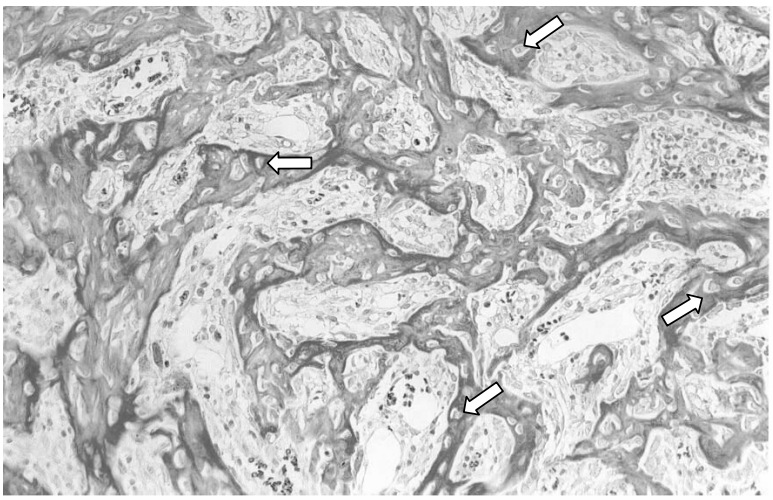


FIGURE 24.1 Woven bone forming within a rat femoral defect. Osteocytes are pointed out with arrows (original magnification 64×).

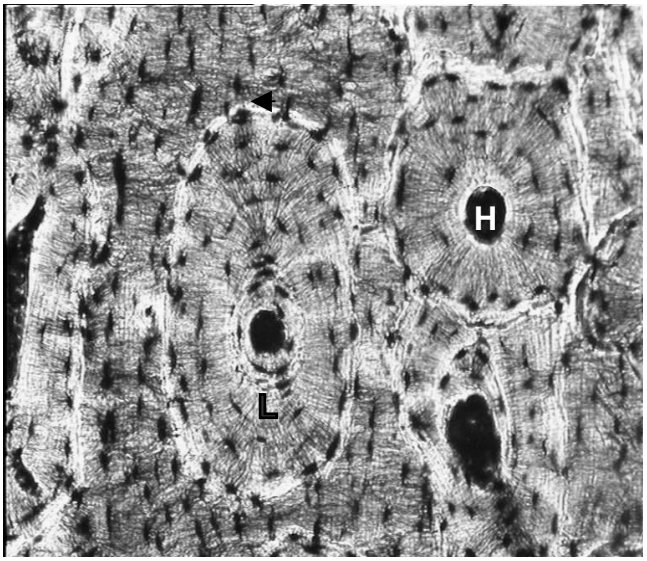


FIGURE 24.2 Human lamellar bone. Notice the organized concentric lamellar structure of an osteon when compared with woven bone. (H, Haversian canal; L, lamellae; original magnification 64×; permanent preparation from Wards collection).

osteocytes.[20] Other smaller capillary-like structures, canaliculi, are also present in the structure,[20,23] extending from the central canal, having the role of feeding the osteocytes.[20] Other canals present in the lamellar bone are the Volkman Canals.[20] These are perpendicular to the central canals, resulting from the branching of these late ones, connecting them to the periosteal surface.[20,23] Other structures, called secondary osteons, can also be found within lamellar bone. Figure 24.2 shows the organization of lamellar bone, in which the presence of several osteons is evident.

24.3.1.3 Cell Biology

Bone formation, maintenance, and resorption are essentially mediated by three cell types: osteoblasts, osteocytes, and osteoclasts.

Osteoblasts (Figure 24.3) arise from osteoprogenitor and mesenchymal stem cells present in the bone marrow and periosteum. As it can be seen in Figure 24.3, these cells are polarized cells, cubic in shape, and are commonly found at the bone surface. They are known to be involved in the synthesis and regulation of extracellular matrix elaboration (ECM) and mineralization.[1,2,25,26] Furthermore, it is also known that basic cellular functions and responsiveness to metabolic and mechanical stimuli demand are maintained through extensive cell–matrix and cell–cell contacts via a variety of transmembranous proteins and specific receptors.[1]

Osteocytes derive from osteoblasts that became incorporated in the newly elaborated extracellular matrix, being enclosed in spaces called lacunae. They maintain direct contact with neighboring osteocytes, osteoblasts, and bone lining cells through cellular processes that are created before and during matrix synthesis.[1,27] In mature bone, these cell processes are contained in channels called the canaliculi. The communication and interaction between neighboring osteocytes is achieved through the establishment of gap junctions.[1,27] This is an absolute need for osteocytes because it is the only way by which they can ensure the access to oxygen and nutrients. Morphologically, mature osteocytes are different from osteoblasts, presenting a stellate or dendritic shape and a 70% volume reduction when compared with the original osteoblast, possessing at the same time fewer organelles. Regarding its function, they are known to be involved in the calcification of osteoid matrix and blood-calcium homeostasis and to be the mechanosensor cells of bone.[20,27]

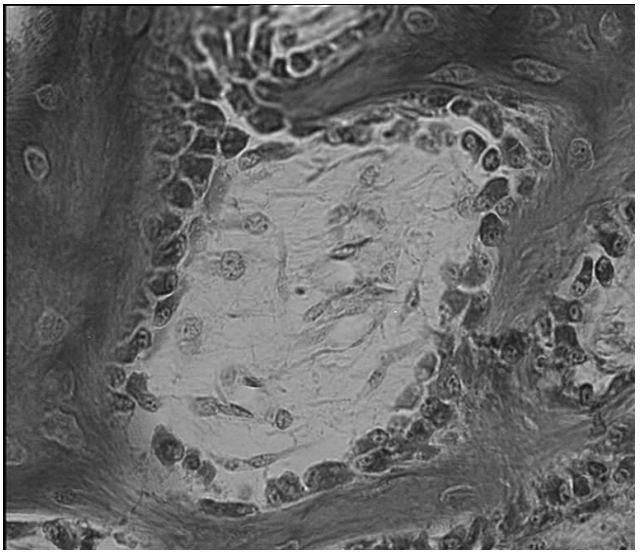


FIGURE 24.3 Osteoblasts around a bone fragment formed during endochondral ossification (original magnification 125×; permanent preparation from Wards collection).

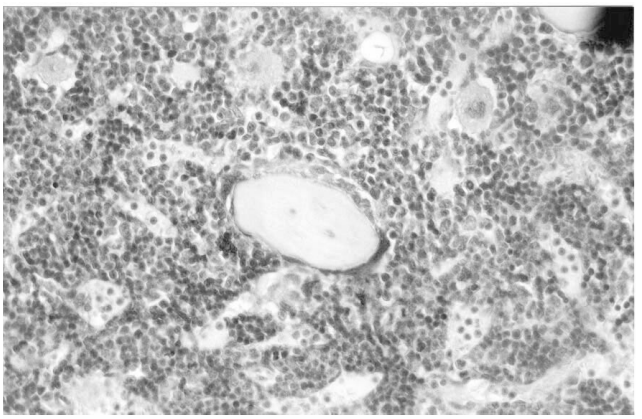


FIGURE 24.4 Osteoclasts actively resorbing a bone fragment during the healing process of a rat femoral defect. Osteoclasts are marked with tartrate-resistant acid phosphatase (TRAP) staining (red) (original magnification, 102.4×).

Osteoclasts (Figure 24.4) are multinucleated polarized cells being involved in the bone remodeling process, belonging to the monocyte/macrophage lineage. Their main function is to resorb mineralized bone.[1,28] For this purpose, they present enriched in intracellular structures such as pleomorphic mitochondria, vacuoles, and lysosomes, as well as alterations, namely at the structural level, in its cell membrane.[1,2,28]

24.3.1.4 Bone Matrix

Bone matrix is composed by two distinct phases, inorganic (mineral) and organic. The latter comprises about 25–30% of the total matrix content, while the first one, constituted by hydroxyapatite, comprises the remaining 65–70% of the matrix.[1]

TABLE 24.1
Components of the Organic Phase of Bone Matrix

Bone Extracellular Matrix Constituent	Function and Properties	Ref.
Collagen I	Provides framework for skeletal structure; matrix calcification	1, 29–31
Byglican	Proteoglycan; affect collagen fiber growth and diameter; involved in the process of matrix mineralization	1, 20, 32
Decorin		
Osteonectin	Glycoprotein; binds Ca^{2+} and collagen; nucleates hydroxyapatite	31, 32
Thrombospondin	Glycoprotein; binds calcium, hydroxyapatite, osteonectin, and other cell surface proteins; mediates cell adhesion in a RGD-independent fashion	20, 32
Fibronectin	Osteoblast attachment to substrate	32
Osteopontin	Sialoprotein; constituent of cement line involved in bone remodeling	27–29, 32–34
Bone sialoprotein	Sialoprotein; constituent of cement line	
Osteocalcin	Skeletal gla protein; late marker of osteogenic phenotype; involved in bone remodeling; it may also be involved in the control of mineralization through its inhibition.	

Several different proteins with different functions constitute the organic phase of the bone matrix. For a simple presentation and better understanding by the reader, the components of the bone organic phase are summarized in Table 24.1.

24.3.2 Cartilage Biology

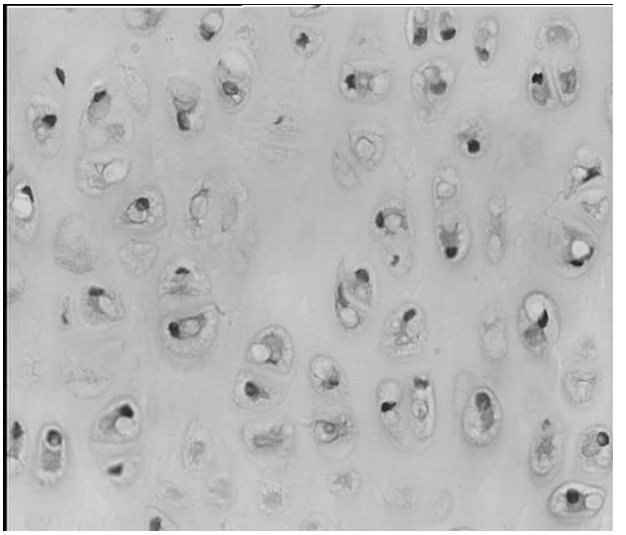
24.3.2.1 General Considerations on Cartilage Structural Biology

There are three different kinds of cartilage in the human body: elastic, fibrocartilage, and hyaline.

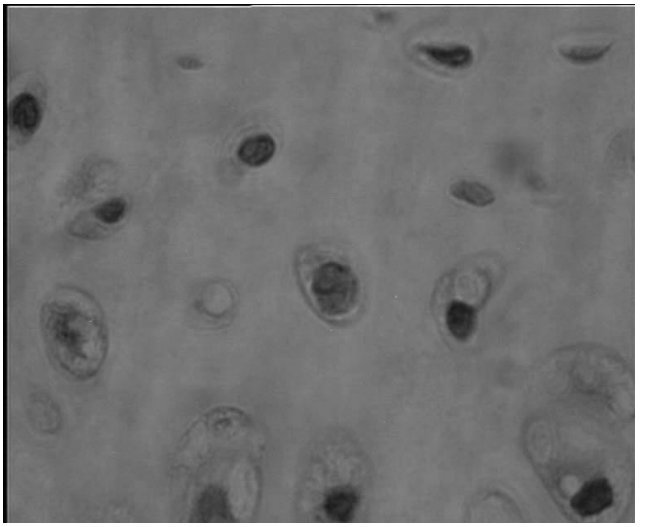
Elastic cartilage is found in the nose and ear and is known for having the presence of elastin in its ECM.[35] *Fibrocartilage* is commonly found in ligaments and tendons and has a higher proportion of collagen in its ECM.[35] *Hyaline cartilage* (Figure 24.5a), which is one of the topics of the present chapter, is commonly found in the joints, such as the knee and ankle. For instance, in the knee joint, a layer of hyaline cartilage covers both femur and tibia. The joint is also encircled by the synovial membrane, which contains the synovial fluid, which provides lubrication and nutrients to the cartilage since its matrix is avascular. Due to its location, it is also called *articular cartilage*. It provides stable movement and can alter its properties in response to differences in loading. In spite of its apparent simple structure, with an avascular matrix, hyaline cartilage possesses properties such as resistance to compression and the ability to evenly distribute the loads when necessary.[35]

24.3.2.2 Articular Cartilage Structural Biology

Opposing to bone, mature articular cartilage is mainly constituted by only one cell type, the *chondrocyte* (Figure 24.5b). These originate from mesenchymal cells during fetal development.[14] In hyaline cartilage, they make up only 1% of the total volume but are extremely important because they do not only produce cartilage matrix, but are also responsible for replacing degraded molecules from the matrix, so the correct size and mechanical properties of the tissue can be maintained.[14] In mature articular cartilage, chondrocytes are completely surrounded by matrix, being located in places called the lacunae. They have a round shape and are unable to proliferate. Two of its marked characteristics are the prominent endoplasmic reticulum and Golgi apparatus,[35] showing the tendency of these cells for protein production. They play a role in sensing the mechanical environment of the cell, since chondrocyte are known to modify matrix properties



(a)



(b)

FIGURE 24.5 (a) Hyaline cartilage and (b) chondrocyte embedded within the collagen matrix with cellular processes in contact with the matrix (original magnification, (a) 125× and (b) 315×; permanent preparation from Wards collection).

when submitted to different loadings.[35] Figure 24.5 is a representative example of the structure and organization of articular cartilage.

24.3.2.3 Articular Cartilage Matrix

Cartilage extracellular matrix not only provides cartilage with the needed mechanical properties, but it is also involved in the regulation of the cellular behavior by providing signals to the entrapped

cells through binding of integrins or other ECM receptors, and by binding, storage, release, and presentation of several growth factors.[36] It is composed of interstitial fluid, several forms of collagen, proteoglycans, and noncollagenous proteins, all of them synthetized by chondrocytes.

Interstitial fluid is an essential part of hyaline cartilage, making up of around 80% of articular cartilage's wet weight. Besides water, it also contains gases, metabolites, and a large amount of cations to balance the negatively charged glycosaminoglycans (GAGs) in the ECM. It is the exchange between this fluid with the synovial fluid that provides the nutrients and oxygen to the chondrocytes. Furthermore, it is also due to its entrapment within the ECM that articular cartilage has the ability of returning to its normal shape after compression.[35]

Collagen types II, VI, IX, X, and XI are the most commonly found in articular cartilage, collagen II being the most abundant.[14,35,37] Due to its structure, collagen II has a higher interaction with water when compared with other collagen types.[35] Together with types IX and XI, it forms an interconnected network of fibrils, which ultimately will provide the needed strength.[3,14,35]

Proteoglycans form a special class of glycoproteins with attached long unbranched and highly charged glycosaminoglycans chains.[38] Aggrecan is the largest proteoglycan present in cartilage.[14,38] Hyaluronic acid, chondroitin sulfate, and keratan sulfate are the most commonly found GAGs associated with aggrecan's core protein.[14] These high-molecular-weight complexes are highly hydrophilic, attracting water and generating a swelling pressure to counter compressive loads, being in this sense extremely important for the maintenance of the mechanical properties and stability of articular cartilage.[38] Decoryn, byglycan, and fibromodolin are other proteoglycans present in cartilage's ECM.[14,15,38] They have one or two GAGs attached to the core protein, and it is believed that they have a role in collagen fiber formation and orientation.[14,38]

As it was said before, cartilage ECM is also constituted by noncollagenous proteins. Cartilage oligomeric matrix protein (COMP) is one of them. It may have a role in stabilizing collagen network and promoting the collagen fibril assembly.[38] Furthermore, it has also been suggested that it can play a role on anchoring chondrocytes to the surrounding matrix and cell interactions.[35] Besides this, cartilage matrix protein (CMP), also known as matrilin-1, cartilage intermediate layer protein (CILP), fibronectin, and fibulin-2 can also be found.[35,38]

24.3.2.4 Articular Cartilage Architecture

Structurally, articular cartilage is divided in four zones: superficial, transitional, middle, and calcified.

The superficial zone, also called zone 1, makes up approximately 10% of the cartilage and determines its load-bearing ability and is the gliding surface of the joint. It is composed of two distinct layers. The upper layer, the lamina splendens, is mainly constituted by collagen fibers and a small portion of polysaccharides and no cells. Bellow this, the second layer is composed of flat chondrocytes tangentially arranged to the articular surface. This particular orientation will confer a higher tensile strength and stiffness to this zone. The superficial zone is also very important for the compressive properties of the tissue. Its removal increases permeability to water, which will later lead to a degeneration of the tissue properties.[3,35]

The transitional zone (zone 2) occupies a larger area than the first one. It is composed of spherical chondrocytes and randomly oriented collagen fibers. Besides having larger collagen fibers, this zone is characterized by having higher proteoglycans content.[3,35]

The middle zone is the biggest, containing the highest content of proteoglycans, the largest diameter for collagens fibers, and the least water. As for the transitional zone, the chondrocytes have a round morphology. However, and opposite to the random distribution found in the transitional zone, chondrocytes are stacked in columns perpendicular to the articulating surface. These cells show higher synthetic activity than those found in the superficial zone. Still, in this area there is another interesting property, the fibers' orientation changes once again, being aligned perpendicularly to the joint surface. These fibers will then extend to a basophilic line of unknown composition, which indicates the beginning of the calcified tissue.[3,35]

The calcified zone joins the hyaline cartilage to the subchondral bone and, together with the superficial zone, is the area of less water content. As it would be expected, it is an area where a significant shear stress happens, due to the interface between hyaline cartilage and bone. Chondrocytes in this area are smaller, when compared to the middle zone, and in some places, it is possible to observe that they are completely surrounded by a calcified ECM.[3,35]

24.4 CELLS FOR TISSUE ENGINEERING OF BONE AND CARTILAGE

The choice of an adequate cell source is essential for the development of a tissue engineering equivalent. These selected sources should allow the isolation of cells in high numbers and the latter should be easily expandable to higher passages, be nonimmunogenic, and have a protein expression rate similar to that of the tissue to be regenerated.[39]

24.4.1 Osteoblasts and Chondrocytes

Autologous osteoblasts and chondrocytes were considered as the ideal cell type in the early years of bone and cartilage tissue engineering. This option was mainly based on the nonimmunogenicity of these cells. However, it is known that this methodology — that is, cells isolated from the patient's tissue to be replaced — had several limitations, mainly due to the low numbers of cells obtained upon isolation, the limited capability of expansion, and loss of phenotypic characteristics after long-term expansion *in vitro*.[39]

Xenogeneic cells (cells obtained from nonhuman donors) could be an alternative to the referred to methodology. This particular path would then solve the problem of low cell number yields. However, the immunogenicity of these cells, the possibilities of the transmission of infectious agents such as virus, and the ethical/social problems related to this issue have limited the enthusiasm for this approach.[39,40]

It is in this context that stem cell biology appears as a valid solution. The knowledge in this research field has grown tremendously in the last years, and although a considerable number of questions are yet to be answered, they can be presented as an alternative to the aforementioned approaches.

24.4.2 Stem Cells

Stem cells are undifferentiated cells with a high proliferation capability, being capable of self-renewal, multilineage differentiation, and hence the regeneration of tissues.[41] However, stem cells have different degrees of potential. The most primitive derive from the fertilized oocyte (the zygote), more precisely from the descendants of the very first divisions.[42,43] These cells are totipotent, because they are able to form the embryo and the trophoblast of the placenta.[42,43] Some days later, these cells start to specialize, forming a hollow ball of cells, the blastocyst, and a cluster of cells called the inner cell mass (ICM), from which the embryo derives. The ICM cells, also known as embryonic stem cells (ES),[42,43] are considered to be pluripotent. They can differentiate into almost all cells that arise from the three germ lines, but not the embryo because they are not able to give rise to the placenta and supporting tissues.[42,43] Finally, we can find multipotent stem cells, also known as adult stem cells (ASC),[42–44] in the fully differentiated tissues. Theoretically, and opposing to ES, these would only be capable of producing a limited range of differentiated progeny, related to the tissue where they are found.[42,43]

24.4.2.1 Embryonic Stem Cells

As stated previously, ES cells reside in the ICM of the blastocyst. They were firstly isolated and grown in culture more than 20 years ago.[45,46] Later on, it was found that when transferred to early

mouse embryos ES cells could give rise to all somatic cell types of the embryo, including the germ line.[47,48] At present, the isolation of ES cells from rodents,[45,46,49] primates,[50] and human beings[51,52] has been reported.

ES cells are characterized by two unique properties[53]: the nearly unlimited self-renewal capability and the capacity to differentiate via precursor cells. Other properties are a high alkaline phosphatase activity, the expression of stage-specific embryonic antigens such as SSEA-1, the expression of germ-line transcription factor Oct-4, high telomerase activity, and the regulation of ES cell self-renewal by cytokines of the IL-6 family.

The differentiation potential of these cells has been reported by several authors,[54–66] in which cardiomyocytes,[54] hematopoietic cells,[55] endothelial cells,[56,57] neurons,[58,59] chondrocytes,[60,61] adypocytes,[62,63] hepatocytes,[64,65] and pancreatic islets[66] were differentiated from ES cells. Of particular interest for bone tissue engineering was the work reported by Buttery et al.,[67] in which osteoblasts were differentiated from ES cells in the presence of dexamethasone. Cells with chondrogenic phenotype were also obtained after exposing ES cells to BMP-2 and BMP-4.[60,61]

However, although they have an enormous potential for biomedical and tissue engineering applications, some questions need to be addressed. To begin with, there is a need to develop methods that allow the direct differentiation of ES cells, their selective differentiation and integration, as well as the tissue-specific function of the ES-cell-generated somatic cells after transplantation.[53] Two other questions that need to be solved: (1) to prove and make clear that ES-cell-derived somatic donor cells are not tumorogenic (it has been known that undifferentiated ES cells give rise to teratomas and teratocarcinomas) and (2) to avoid the immunological incompatibility between ES-cell-generated donor cells.[53] This last point could be solved by using the somatic nuclear cloning transfer (SCNT).[42] However, this will only increase the critics' objections to their use and would raise even more the ethical and social questions, which are probably the most difficult barrier to overcome, in order to use ES in regenerative medicine.

24.4.2.2 Adult Stem Cells

ASCs reside in the fully differentiated or adult tissues. Up to now, ASCs were found in the bone marrow,[68] periosteum,[69,70] muscle,[71] fat,[72] brain,[73,74] and skin.[75]

The bone and cartilage tissue engineering field has shown a special interest in the stem cells located in the bone marrow, known as mesenchymal stem cells (MSC). These cells were firstly described by Friedenstein[76] and were later named by Arnold I. Caplan with the name by which we know them today.[77] In 1994 the same author[78] described that these cells, when placed in adequate culture conditions, could be differentiated to cells with mesenchymal origin and give rise to bone, cartilage, fat, muscle skin, tendon, and other tissues of mesenchymal origin, through what was called the "mesengenic process."

In recent studies published by several authors,[68,79–83] these cells were able to develop into distinct terminal and differentiated osteoblasts[68,79–81] and chondrocytes.[68,81–83]

Besides their differentiation potential, MSCs present other important properties. As described by Bruder et al.,[84] they can be extensively expanded. Pittinger et al.[68] also showed that in increased number of passages did not spontaneously differentiate. Furthermore it has been suggested that these cells may possess immunosuppressive effects which may render them either "immune privileged" or perhaps immunosuppressive roles *in vivo*, which would make them suitable for allogeneic or xenogeneic transplantation.[85] However, this subject needs to be further investigated.

There are no universal markers to identify MSCs.[86] However, a series of stem cell surface markers that may allow for an easier isolation and characterization of MSC populations in culture has been described recently. For instance, antibodies SB10, SH-2, SH-3, and SH-4 were found to bind to MSCs.[87–90] Pittinger et al.[68] described that human MSCs were shown to express a homogeneous (> 98% purity) nonhematopoietic phenotype. Furthermore, it was also found that they are positive for SH-2, SH-3, CD71, CD44, and CD29 receptors.[68]

Although MSCs have several advantages regarding their use for tissue engineering, there are still some issues that need to be addressed. For instance, it is known that the percentage of MSCs present in the bone marrow is very low (1 in each 100,000 cells)[78], which would make the expansion time consuming. New expansion methods can be the solution. Baksh et al.[91] have recently described the expansion of the nonhematopoietic fraction of cells by using a dynamic rotating environment. By doing so, and using the appropriate cytokine "cocktail," the expansion rates were increased when compared to standard culture techniques.[91] The differentiation capability of donors from different ages also needs to be addressed. It has been shown that the numbers as well as the differentiation potential of MSCs was somewhat diminished when these were isolated from elderly patients.[92–94] Finally, like in the ES cells, the knowledge regarding the mechanisms and pathways that lead to the final osteogenic and chondrogenic differentiations is still scarce.

Overall, it can be said that, for now, MSCs present more advantages, or at least fewer drawbacks to overcome, than ES cells for use in bone tissue engineering. For instance, the first are already in clinical trials for certain applications, including bone tissue engineering,[95] while the latter still have a long way until they reach that stage.

24.5 GROWTH FACTORS

Growth factors are secreted by many cell types and function as signaling molecules. The binding of a growth factor to its receptor initiates intracellular signaling that will lead to different events such as the promotion or prevention of cell adhesion, proliferation, migration, and differentiation by up-regulating or down-regulating the synthesis of proteins, including growth factors and its receptors.[10,97] Hence, these molecules are essential for tissue formation and play an important role in tissue engineering.

As other tissues, bone and articular cartilage do also possess several growth factors. Of these, bone morphogenetic proteins (BMPs), transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), insulin-like growth factor I and II (IGF I/II), and platelet-derived growth factor (PDGF) are the most common and those that have realistically been proposed for bone and cartilage tissue engineering applications.[14,96–103]

BMPs and TGF-βs are the two most well-known groups within the growth factors for bone and cartilage regeneration. Both of them are included in the TGF-β super-family by virtue of their similarities in protein structure and sequence homology.

BMPs were first described by Urist[104] and are commonly entrapped within bone and cartilage matrix. They are also expressed during the early stages of fracture healing.[99] As with growth factors, their expression rates vary during the healing process and they manage to up- or down-regulate the expression of other BMPs.[100] It also is known that they can intervene on the expression of other growth factors, such as TGF-β, or vice versa.[96] Regarding cartilage biology, BMP-2, -4, -6, and -7 appear to enhance the chondrogenic phenotype, causing an increase in collagen II and proteoglycan synthesis and a reduction in the synthesis of collagen I.[14,60,61] Regarding the osteogenic phenotype, their main role is to recruit mesenchymal stem cells to the healing site, and then differentiate them into the osteogenic lineage. The mechanisms by which they act on the MSCs are not yet completely understood, but it is known that, for instance, BMP-2 plays an important role on the expression of the osteogenic markers such as alkaline phosphatase and osteocalcin through the mitogen-activated protein kinase (MAPK) pathway.[105] At the same time, it is probable that they are also involved in the expression of the nuclear transcription factor Cbaf-1/Runx2.[96] BMPs have already been used in bone regenerative preclinical and clinical trials.[106,107] In spite of the fact that good results were achieved, a problem arose from those experiments; the therapeutic dose varied as much as 100-fold, making difficult the task of finding an optimal concentration for human clinical trials.[107]

Generally speaking, the biological actions of TGF-β are very diverse. It has been shown to stimulate cellular proliferation *in vitro* and to promote cellular hypertrophy and differentiation.[108] TGF-β has also shown to block or initiate cellular migration or differentiation.[108] It stimulates

osteoblast-like cells to proliferate and promotes collagen production *in vitro*.[101] *In vivo* studies have shown that TGF-β increases callus formation on the fracture healing site.[109] Regarding cartilage, TGF-βs, namely 1, 2, and 3,[68,81–83,101,110,111] have shown to potentiate chondrogenic differentiation, through the activation of several signaling pathways, as reviewed by Grimaud et al.[102] However, because it is involved in several cellular events, it is crucial to control its bioavailability as a therapeutic agent.[108]

Both IGF genes are expressed by skeletal cells, and though IGF-I and -II have similar effects on bone metabolism, IGF-I is more potent than IGF-II.[112] Upon injury, they are found in the fracture healing sites, and it is known that they stimulate type I collagen synthesis and increase matrix apposition rates.[96,112] In addition, they maintain collagen integrity in the bone microenvironment by decreasing collagen synthesis or by decreasing the expression of interstitial collagenase by the osteoblasts.[96,112] In chondrocytes, IGF-I is also the most used. It has been reported to predominantly stimulate matrix synthesis with a minor effect on mitotic activity in articular chondrocytes.[113,114] Furthermore, it has also been shown that it can prevent apoptotic cell death of chondrocytes[115] and hence can be a useful tool to prevent cartilage degeneration.

Besides these, other growth factors have the potential to be used for bone tissue engineering. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and is expressed in a variety of highly vascularized tissues.[116,117] It is commonly found in bone fracture healing sites and in the growth plate and regulates vascularization through the recruitment of endothelial cells to the healing site.[96,118] It also plays an important role in the regulation of the interaction between osteogenesis and angiogenesis.[118] Fibroblast growth factors (FGFs), namely FGF-2, are yet another cytokine involved in the bone-remodeling process. It is believed that they are involved in the regulation of the maintenance of the delicate balance between bone-forming cells and bone-resorbing cells.[96] They also promote the development of new blood vessels[96] and play a role in the stimulation of the osteogenic phenotype through the activation of the Cbaf-1/Runx 2 nuclear transcription factor.[119] FGFs are also involved in the chondrogenic process, namely FGF-2 and FGF-18.[120–122] The first one helps to maintain the reexpression of the chondrogenic phenotype after long-term expansion *in vitro*, while the second acts as a trophic factor for chondrocytes and MSCs.[120–122] Finally, PDGF can also play a role in the bone regenerative process. It is produced by osteoblasts, platelets, and monocytes/macrophages, and it is believed to play a role in the migration of MSCs to the wound healing sites.[123]

24.6 BIOREACTORS IN TISSUE ENGINEERING OF BONE AND CARTILAGE

Physical forces, such as dynamical loading, fluid flow, and hydrostatic pressure, have been implicated in the regulation of bone and cartilage matrix metabolism *in vivo* as well as in *in vitro* systems.[124–130] These mechanical stimuli that affect cellular functions are probably transmitted to cells by forces affecting the cellular microenvironment. It has been shown that osteocytes are the mechanosensor cells of bone,[20,27] and chondrocytes are connected to the surrounding matrix by focal adhesions.[131] These focal adhesions not only mediate cell adhesion, but they also serve as mediators of signal transductions through the propagation of extracellular stimuli.[3]

The most widely used culturing technique in tissue engineering studies is static culturing, which is often characterized by nonhomogeneous cell distribution, confining the majority of the cells to the outer surfaces of the scaffold, which in turn results in a nonhomogeneous distribution of the *in vitro* generated extracellular matrix.[129,130,132] In this sense, it is essential to develop new culturing techniques and bioreactors that will allow the culturing of osteogenic and chondrogenic cells with scaffolds under the appropriate conditions.

In order to overcome this limitation, several culturing systems, consisting basically of growth chambers equipped with stirrers and sensors that regulate the appropriate amounts of nutrients, gases, and waste products, have been developed.[128–130,132–135] These systems, so-called bioreactors,

may have different designs attempting to achieve one or more of the following objectives: (1) maintain a uniform distribution of cells into the 3-D scaffolds, (2) provide adequate levels of oxygen, nutrients, cytokines, and growth factors, and (3) expose the cultured cells to mechanical stimuli. Furthermore, experiments involving *in vitro* bioreactor culturing can also be designed to study the effects of specific biochemical and physical signals involved in cell/tissue development and function, providing useful information on the processes that lead to the formation of 3-D tissues starting from cells/material tissue-engineered constructs.[132]

Bioreactors are also the focus of manufacturing technology for tissue-engineered products because they represent a chemically and mechanically controlled environment in which a tissue-like construct can be grown.[136]

There are several types of bioreactors currently available, which can be grouped in three main types, namely the spinner flasks,[129] the rotating bioreactors,[129,130,132,137] and the flow perfusion culture systems.[124,128] The type and the specific functional design characteristics of these bioreactor systems are determined by the dimensional and functional requirements of the tissue to be substituted/regenerated as well as by the cell–scaffold system used.

In the first one, scaffolds are attached to the needles hanging from the lid of the flask, and convective forces generated by a magnetic stirrer bar allow continuous mixing of the media surrounding the scaffolds.[129] The second one is characterized by the maintenance of the cells in a microgravity state,[129,130,137] also presenting a low fluid shear stress. Finally, in the third type of system, cell–scaffold constructs are exposed to a continued flow of culture medium through its structure, which allows a constant nutrition and prevents unphysiological accumulation of metabolic products.[128,129,138] Of the three systems, the one that has presented better results so far is the last one, mainly due to the fluid flow shear stress, hydrostatic pressure, and an accurate spatial distribution of the cells throughout the scaffold structure.[129]

24.7 ANIMAL MODELS

The appropriate choice of an experimental model to assess the feasibility of a determined tissue engineering concept is critical to the success of the preclinical studies. The criteria associated with the choice of an experimental model must be related to its functional application and often to the expected commercial market of the bone/cartilage tissue-engineered construct.[139] The following properties are found to be essential when choosing an animal model[140]:

1. It must mimic, as much as possible, the clinical setting such that it is biologically analogous and recognizable as an appropriate challenge to human physiology.
2. The bone/cartilage defect must fail to heal unless it is treated with the tissue engineering strategy under study.

In the early phases of a particular research program, smaller animals can be used just to test the proof of concept that is under development. However, in the later and more advanced phases of the studies, the evolution to more advanced models and larger mammals is needed, mainly in those cases where the human clinical conditions need to be matched.

Ectopic models are often used as a first approach for *in vivo* testing within the field of bone/cartilage tissue engineering.[141] *Ectopic* means that the constructs are placed in areas other than bone or cartilage sites. Usually they are placed in one of the following locations[140,141]: subcutaneously, intramuscularly, intraperitoneally, and in the mesentery. The first one is the most popular and has been used by several authors to assess the feasibility of their scaffolds/cells constructs in an *in vivo* environment.[142–144] These models are particularly useful to determine whether a scaffold has an adequate porosity for tissue ingrowth and vascularization and to assess whether it triggers inflammatory reactions. It is also used to assess ectopic bone/cartilage formation of scaffolds loaded with growth factors and the ability of TE constructs composed by osteo-

genic/chondrogenic cells and scaffolds to induce bone and cartilage formation. For the later objective, athymic nude mice are commonly used due to the lack of immunogeneic response when using xenogeneic cells (e.g., human MSCs).[107]

These ectopic models are particularly useful for the initial stages. However, after testing the concepts in these smaller and simpler models, the researchers should advance to models where it is possible to create defects that mimic the clinical situations to be solved.

24.7.1 Animal Models in Bone Tissue Engineering

Regarding bone defects, there are mainly four types of defects: calvarial, long bone or mandibular segmental, partial cortical (cortical window, wedge defect, or transcortical drill), and cancellous bone defects.[140,141] Although rats are still used for these purposes, rabbits are commonly the chosen animals. However, it should be reminded that in some cases, the results obtained with rabbits will not be translated to humans, namely due to the high metabolism and fast healing capability of these animals. Dogs and sheep are also used, but to a lesser extent.[141] Regarding these models, one aspect should be taken in account, the age of the used animals is important. It has been shown that immature animals heal faster than mature ones, and hence if used, they can be misleading.[145] Another issue is the size of the defect. Critical size defects should be used in order to demonstrate the full feasibility of the tissue-engineered constructs. A critical size defect (CSD) is defined as the smallest size of a defect which does not heal spontaneously when left untreated for a certain period of time.[141]

For initial *in vivo* assessments, the cancellous bone defect in rats can also be used, allowing the researcher to evaluate the scaffolds behavior, namely bone ingrowth, osteoconduction, and inflammatory reaction, while implanted in a bone environment.[140]

If the objective is to regenerate craniofacial defects, the rabbit calvarial model can be used. This model is very popular and appropriate for the following reasons[141]: (1) the calvarial bone is a plate which allows the creation of a uniform circular defect that enables convenient radiographical and histological analysis; (2) the calvarial bone has a good size for easier surgical procedure and specimen handling; (3) no fixation is required because of the good supports by the dura and the overlying skin; (4) the model has been thoroughly used and studied and is well reproduced; and (5) it is relatively economical compared with dogs. A critical size defect (CSD) for the rabbit calvaria model is 15 mm.[141] The rat calvarial defect can be used, if rabbits are an expensive option. In this case, the CSD is 8 mm. However, there is a major concern about this model, which is the fast healing ability of the rat.

In the case of long bone segmental defects, the rabbit radial model is also popular and can be used for the following reasons[141]: (1) the radius bone is tubular, which allows the creation of segmental defects that enables convenient radiographical and histological analysis; (2) no fixation is required because of the support of the ulna; and (3) it is relatively economical. In this case, a 15-mm defect is defined as CSD.

If the researcher wants to mimic a clinical application and place the TE construct under a load-bearing condition, segmental defects can be performed in the femur of rabbits. However, in this particular approach, an internal or external fixation device will be required.[140]

Pigs or sheep models are other possible options. However, their use is rare, mainly due to the high costs involved.

24.7.2 Animal Models in Cartilage Tissue Engineering

For cartilage repair studies, the chosen animals are often rabbits. As for the bone models, a special emphasis should be put on the choice of the adequate species, as well as the age of the animals. Another important issue is the age of the defect. The majority of the patients usually have a long history of complaints, before they are treated for the damaged joint, which means that the cartilage matrix itself already presents a certain degree of degradation. Being so, it can be said that the regenerative potential between those and a recent defect, like the one done in animal models, is

different and hence could affect the final interpretation of the results and consequent clinical application.[146] The location of the defects is also important, mainly due to the mechanical loading that will be imposed to the tissue engineering construct. For instance, a defect created on the distal femoral surface is considered to be weight bearing, while a defect in the intercondylar groove is thought by some to be partial-weight bearing.[146]

There are three different types of cartilaginous defects that can be studied and evaluated[146]: (1) partial-thickness cartilage defects (the defect is located entirely within and surrounded by cartilage); (2) full-thickness cartilage defects, which extend down to the subchondral bone but do not penetrate it; and (3) osteochondral defects, which extend into the subchondral bone. Creating a partial-thickness defect is somewhat troublesome and challenging, because articular cartilage thickness varies from species to species, and no animal model is comparable to humans with these regard.[16,146] Because of these, Hunziker has proposed a virtual partial-thickness defect that would be of a similar dimension to a human partial-thickness defect.[146] In this model, the floor and walls of the defect would be made impermeable, by using a membrane, to blood-borne cells and signaling substances derived from the subchondral bone tissue. However, in spite of being an interesting concept, this model still needs to show its functionality.

Histological scoring systems are often used in order to analyze the outcome of the *in vivo* cartilage regeneration assays. As referred by Reinholz et al.,[146] the two that are most frequently used are the Pineda[147] and the O'Driscoll[148,149] scoring systems. Both of them are reliable semi-quantitative scoring systems with good correlation**.**

24.8 CONCLUDING REMARKS

Tissue engineering of bone and cartilage is in fact a promising field to overcome the shortcomings of the existing therapies for bone/cartilage replacement/regeneration. As it was said in the beginning, the first step is to develop an adequate matrix, a scaffold, to allow cell growth. However, after this goal is successfully achieved, it cannot be expected that nature takes over and bone and cartilage regenerate only by the simple implantation of the scaffold. It is also important to understand bone and cartilage biology, not only at the cellular level but also at the molecular level. Furthermore, it is important to understand how growth factors act on cells, what effects are caused by them, and hence how we can use them within this fascinating field. Finally, it is necessary to develop and use adequate animal models that not only allow the researcher to verify the developed concepts but at the same time exactly mimic (as much as possible) the situations that are pretended to be regenerated. In sum, besides a profound knowledge of materials science, it is also necessary to have a deep understanding of the biological components so that tissue engineering can be in the near future a solution, and not only a running promise, for the existing problems of bone and cartilage replacement/regeneration.

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25 Tissue Engineering of the Liver

Y. Murat Elcin

CONTENTS

25.1 INTRODUCTION

Liver failure, among other diseases, remains one of the major causes of mortality in the contemporary world. As a consequence of the increase in liver disease incidence, the shortage of donor organs, and the demand for sophisticated transplant teams, interest in hepatocellular therapies is gradually on the rise. These therapies can be categorized under three headings, namely extracorporeal bioartificial liver devices, cell transplantation, and tissue engineering.

Bioartificial liver support systems are extracorporeal devices containing viable liver cells; they are basically used as hemoperfusion chambers where the blood is separated from the hepatocytes by a porous immunoisolatory membrane.[1,2]

In a number of inherited hepatic disorders, hepatocyte transplantation has been proposed as a tool for liver-directed gene therapy.[3,4] This approach can be implemented by a variety of configurations, such as using cell suspensions, cells attached to substrates, and cells encapsulated in microsphere- or hollow-fiber diffusion chamber-like systems.

TABLE 25.1
Cell Sources Used in Experimental or Clinical Hepatocellular Approaches

Cell Sources	Explanation	Ref.
Primary cells	Human cells (low availability, low functional stability)	4, 13, 14
	Porcine cells (high availability, low functional stability, xenogenic)	15–17
Cell lines	Immortalized cells (unlimited source): human, spontaneous, HH 25; porcine, pRSVneo, HepLiu; human fetal, SV40T, Yoon	19, 20, 23
	Tumor-derived cells: human hepatoblastoma, Hep G2; human hepatoblastoma, C3A; human hepatoblastoma, HuH6	21, 22, 25
Stem cells	Progenitor/transdifferentiated	26–30
	Embryonic	31–34

Tissue engineering has emerged as an alternative approach to treat the loss or malfunction of a tissue or organ and has the advantage of not having the limitations of the current orthodox therapies.[5,6] Basically, the concept of this technology is the transplantation of constructs consisting of endogenous stem/progenitor cells, established cell lines, or primary hepatocytes, grown *ex vivo* within predesigned scaffolds made up of exogenous 3-D extracellular matrices. The scaffold employed to guide the functional tissue development will eventually break down, leaving only the cells and the stroma that they produce in the body. The other approach of engineering tissues is the acellular one. This approach has emerged with the new findings in stem cell biology and cell signaling associated with phenotypic induction. The idea is that by using appropriate biomaterials and the body's own reservoir of stem cells, it may be possible to develop functional liver tissue organoids suitable for replacement of the diseased tissue.[7] For the time being, engineering of implantable liver constructs is largely experimental and is still to overcome significant hurdles before it becomes a viable clinical modality. Development of an engineered, fully functional liver tissue requires the formation of the vascular, biliary, and parenchymal structures, including the stellate and other cells, with precise timing, which seems to be a very difficult task. Engineering of the liver tissue is the focus of this chapter; however, other hepatocellular therapies will also be discussed.

25.2 CELL SOURCES FOR HEPATOCELLULAR THERAPIES

The liver is a large parenchymal organ with several separate lobes and two separate afferent blood supplies. Hepatocytes, bile duct epithelium, stellate cells (formerly called Ito cells), Kupffer cells, vascular endothelium, fibroblasts, and leukocytes are the main cell types of the liver. Hepatocytes are responsible for most organismal liver functions.[8,9] Stellate cells synthesize the extracellular matrix proteins and many hepatic growth factors and store vitamin A.[10] Kupffer cells are the resident macrophages of hematopoietic origin, capable of replicating within the liver itself. Oval cells are hepatic progenitors found in the regenerating liver.[11,12]

The choice of the cell type in hepatocellular therapies is of great importance. On the other hand, full complement of cellular functions required to replace the liver and positively affect clinical outcomes has not yet been determined. Hence, functionality of cellular devices is determined by markers of liver-specific functions, including metabolic and synthetic functions, phase I and II pathways of detoxification, and biliary excretion. Table 25.1 summarizes the potential cell sources that can be used in hepatocellular therapies.

25.2.1 Primary Hepatocytes

Primary hepatocytes are the most common cellular component in current engineered therapies. Primary human cells are the preferred source,[4,13,14] but like whole organs, they are in limited supply.

Most bioartificial liver support devices undergoing clinical evaluation use porcine hepatocytes which are readily available.[15–17] Porcine hepatocytes do express a stable albumin synthetic function, but show a decline in cytochrome P-450 activity under standard culture conditions.[18] In general, primary hepatocytes require specific microenvironmental cues to maintain hepatic phenotype *in vitro*.

25.2.2 Hepatocyte Cell Lines

The development of highly functional hepatocyte cell lines for use in cellular therapies seems to be the strategy to overcome the growth limitations of primary cells. Hepatic cell lines have been derived from retroviral transduction of the simian virus 40 tumor antigen gene (SV40 tag),[19,20] from liver tumors, as in the case of HepG2 and C3A,[21,22] and from collagen gel sandwich cultures by spontaneous immortalization.[23] In general, immortalized hepatocytes are less sensitive to important physiologic cues.[24,25] The risk of oncogenic factor transmission to the host, especially in cell transplantation and tissue engineering therapies is a major concern. The cell lines should be evaluated on the basis of liver-specific function and safety. The cell lines that have shown therapeutic effects are the cell lines or primary cultures derived from hepatocytes themselves.

25.2.3 Stem Cells

Stem cells are known to play a major role during development and organogenesis. They are self-renewing cells with the potential to differentiate into specialized cells. Potential stem cell sources for use in cell-based therapies are embryonic stem cells, hepatic stem cells (adult liver progenitors), and transdifferentiated nonhepatic cells.[35–38]

25.2.3.1 Adult Stem Cells

The liver is classified as a conditionally renewing organ, and all hepatocytes are proliferatively quiescent with only 0.3–0.5% dividing.[39] However, following injury, the resting hepatocytes are able to function as unipotential stem cells.[26,40] Liver fabricates new functional structures by processing signals that are preprogrammed into cells.[27] Liver stem cells are precursors of the two epithelial liver cell types, the hepatocytes and the bile duct epithelial cells. Oval cells are the descendants of the stem cells and are found in the portal and periportal regions in experimental animals within days of the liver injury. Recent studies suggest that oval cells can differentiate into bile ductular cells or hepatocytes to allow repopulation of the injured liver. Interrelationships between the putative stem cells of the liver are summarized in Figure 25.1.

Recently, Takimoto et al. (2003) have developed a novel strategy for liver tissue engineering that involved the use of putative liver stem cells.[42] In this approach, a cone-shaped acellular collagen-polypropylene composite scaffold was inserted into the rat liver with minimal bleeding; after 3 months desmin-positive staining hepatic stellate as well as OV-6 staining hepatic oval cells were observed inside the scaffolds. After 6 to 8 months, the cells inside the scaffold had formed rudimentary nodules that stained positive to alpha-fetoprotein, demonstrating the existence of immature hepatocyte-like cells.

Hepatic progenitors are proposed as ideal cells for use in liver cell therapies given that they have the ability to expand extensively, can differentiate into all mature liver cells, possess minimal immunogenicity, are cryopreservable, and have the ability to reconstitute liver tissue when transplanted.[43] Although strategies are available to purify and culture hepatic oval cells,[44] a detailed understanding of the growth factors and cytokines that are important in their survival and differentiation is yet to be reached. In the future, hepatic stem cell transplants or hepatocytes derived from them may be used to treat acute liver failure cases.

Recent reports that hematological stem cells may also contribute to liver regeneration[28] bear implications to hepatocyte transplantation and liver tissue engineering in human beings. Depending on the mode of injury or the model, hematological stem cells can regenerate hepatocytes directly,

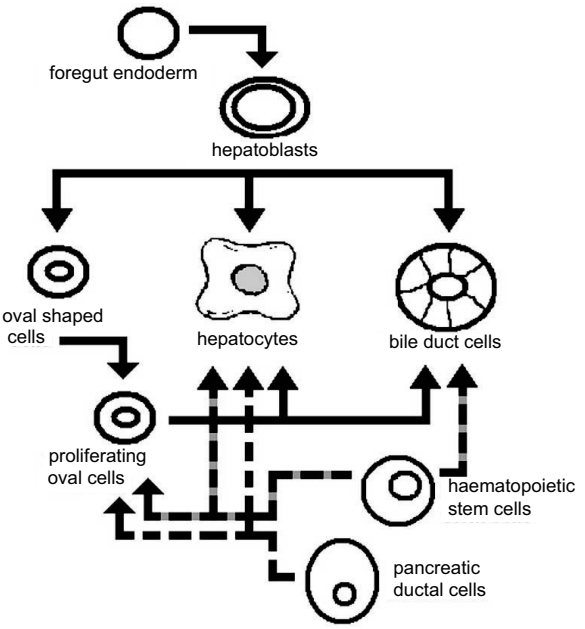

FIGURE 25.1 Proposed interrelationship between the putative stem cells of the liver.

or through an oval cell intermediate.[29] Tissue engineering biopolymer scaffolds enriched with signaling complexes but lacking cells can be designed to attract the host stem or progenitor cells from the implantation site or from the circulating blood.[7]

25.2.3.2 Embryonic Stem Cells

Embryonic stem (ES) cells derived from the inner cell mass of mammalian blastocyst are known for their potential to maintain an undifferentiated state through an extended number of passages.[45] With the appropriate stimulation, ES cells differentiate into various lineages of all three germ layers and thus can serve as a powerful resource of cell populations for cell transplantation and tissue engineering.[6,46]

ES cells have been reported to differentiate *in vitro* into cells of endodermal origin that express endodermal and hepatic markers.[31] Yamada et al. (2002) have shown the *in vitro* differentiation of mouse ES cells into hepatocyte-like cell clusters that can form a three-dimensional structure capable of demonstrating the cellular uptake of the organic test substance, indocyanine green.[32] Additionally, Choi et al. (2002) have shown the differentiation of mouse ES cells into functional hepatocytes in an immunosuppressed nude mouse model.[33]

Chen et al. (2003) have studied the *in vitro* differentiation of rhesus ES cells and the formation of tissue-like structures in 3-D collagen matrixes, as well as the role of intercellular interactions in this process. In this study, they found that in the presence of feeder cells or exogenous cytokines, ES cell differentiation could be directed into a particular lineage, including an accompaniment by the formation of tissue-like structures.[47]

Recently, scientists from Geron Corporation (Menlo Park, CA) have discovered methods to produce uniform populations of hepatocytes derived from human ES cells.[34] The human ES cell-derived hepatocytes express albumin, alpha-1-antitrypsin, glycogen, and other proteins, as well as certain Phase 1 and Phase 2 drug-metabolizing enzymes. Apart from the advantage of the availability of an unlimited human hepatocyte source and thereby promising potential for drug screening

TABLE 25.2
Routes of Hepatocyte Transplantation

Implantation Site	Ref.
Peritoneal cavity	55–58, 65, 66
Spleen	51, 62–65
Liver	4, 13, 60, 61, 65
Mesentery	67
Subcutaneous tissue	65

applications[34] (e.g., hepatic toxicity and metabolic profile of new drugs), studies on BAL support and liver tissue engineering may also benefit from this technology.

25.3 HEPATOCYTE TRANSPLANTATION

Hepatocyte transplantation has been proposed as, primarily, a tool for the reestablishment of liver function by liver-directed gene therapy in a number of inherited hepatic disorders.[48] This approach has been used for nearly 20 years in experimental studies[26,49,50] and has started to move to clinical settings in the last decade.[4,13,14,51] It is estimated that approximately $0.5–1.0 \times 10^{10}$ hepatocytes (2–5% of the liver) suffice for this type of therapy. It has been shown that the majority of the (> 90%) host hepatocytes can be replaced by a small number of donor cells using a therapeutic liver repopulation process that is similar to repopulation of the hematopoietic system after bone marrow transplantation.[52] Liver repopulation occurs when transplanted cells have a growth advantage in the setting of damage to recipient liver cells.

Injection of free hepatocyte suspensions gives rise to differing results, variations being related to the lack of cell support and to the transplantation site.[53] The use of encapsulation devices[54–57] or microcarriers[58,59] has emerged as alternatives to transplant hepatocytes. Hepatocyte transplantation sites include the liver,[4,13,60,61] the spleen,[51,62–65] the peritoneal cavity,[55–57,66] the mesentery,[67] and the subcutaneous tissue[68] (Table 25.2). The liver has proved to be the optimal site for transplanting hepatocytes (through the portal vein) in many cases; however, this route may impose problems such as hypertension, and necrosis by the formation of cell aggregates in the sinusoids, portal branches, and central veins.[60]

25.4 BIOARTIFICIAL LIVER (BAL)

The treatment of acute liver failure has evolved to the current concept of hybrid bioartificial liver (BAL) support, since artificial systems have not proved to be efficient.[69–71] Hepatocyte-based bioartificial liver support systems represent a promising approach for temporary replacement of normal liver function. BAL systems can be used to treat patients with acute fulminant hepatic failure (to support liver function) for liver regeneration, to stabilize the critically ill patients waiting for a transplant, or to provide a support for chronic liver failure patients during acute exacerbations. In the last decade, BAL device technologies have been on a steady progress and the implementation issues now focus upon the scaling of system size for human support.[18] Limited availability of human liver cells remains a serious constraint to the use in BAL devices.[72,73] Due to large yield of viable and functional hepatocytes, and to anatomical and physiological similarities between the species (pigs and humans), porcine hepatocytes represent an attractive source of cells for BAL devices. The other cell source used in BAL devices is the cell lines.[19,21,23,26] A BAL bioreactor design should insure the required oxygen tension of the large numbers of hepatocytes for optimal viability and metabolic output. Otherwise, the consumption can lead to local domains where a significant fraction

of cells are either hypometabolic or dead from low oxygen tension,[74,75] such as in multicellular systems or high-density cell packaging topologies.[76–78] Alternative topologies to attachment-based BAL designs include spheroid-microcarrier[75] and hollow-fiber[71] cell packaging models.

25.5 TISSUE-ENGINEERED CELLULAR CONSTRUCTS

Previous findings suggest that, when provided with the appropriate environment, dissociated liver cells will reassemble *in vitro* into structures that resemble the original tissue.[79–81] In principle, it may be possible to engineer the liver, if the exogenous extracellular matrices can be designed to bring hepatocytes into contact in a suitable 3-D environment, provide mechanical support until the newly formed liver organoids are structurally stabilized, and bring specific signals that guide the expression of genes of the liver cells forming the neotissue.[68,82] This approach has similarity to cell transplantation, in that hepatocytes are transplanted to perform liver functions. However, anchorage-dependent hepatocytes are immobilized on scaffolds, encapsulated in aggregates, or cultured *ex vivo* to form liver organoids and surgically transplanted.

25.5.1 Synthetic/Biological Extracellular Matrix Analogues

Three-dimensional scaffold architecture and chemistry clearly play a role in hepatocyte survival, morphogenesis, and function. Tissue engineering constructs are designed as the attachment substrate and a delivery vehicle for transplanted cells at specific sites of the body. A large surface-area-to-volume ratio is desirable that permits the delivery of a high density of cells. Additionally, the scaffold design should incorporate signals that affect hepatocyte regulation, function, and reorganization. The construct should provide temporary mechanical support to withstand *in vivo* forces until the engineered tissue has sufficient mechanical integrity to support itself. The fate of the seeded cells strongly depend on the cell surface receptors interacting with the support material, interactions with surrounding cells, and the growth factors of the environment (Figure 25.2). Cell-adhesion peptides and growth factors can be incorporated into the synthetic ECM, or mechanical stimulation can be used to manipulate these factors.[1,2]

Liver tissue engineering constructs can be fabricated both from materials of synthetic and biologic composition with varying chemistry and topography. These include biodegradable synthetic polyesters (e.g., α-hydroxy acid polymers and copolymers),[82–88] semisynthetic polysaccharides (e.g., chitosan, hyaluronic acid, and alginate),[68,80,89–92] collagen,[93] complex biomatrices[94] (e.g., biomatrix from connective tissue, amniotic membrane extracts, and tissue extract enriched in embryonic matrix components), and self-assembling peptides.

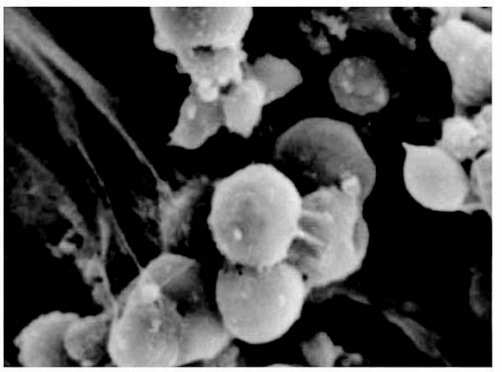

FIGURE 25.2 Cells seeded on a three-dimensional scaffold composed of a synthetic polyester. Interconnected macropores of these scaffolds permit easy access to cell–cell interactions and cell migration.

Homo- and heteropolymers of poly(glycolic acid) (PGA) and poly(lactic acid) (PLA) and their blends have been used to fabricate liver tissue constructs and were found suitable to extended hepatocyte culture with stable differentiated functions.[84] Johnson et al. (1994) have transplanted hepatocytes on filamentous PGA sheets into the mesentery of UDP-glucuronyl transferase-deficient Gunn rats and observed bilirubin conjugates in bile profiles of animals.[67] Carbohydrate-modified poly(ethylene oxide) has been used to promote hepatocyte adhesion through the asialoglycoprotein receptors.[79] Gutsche et al. (1996) have evaluated heparin and lactose-grafted foams for their ability to hepatocyte adhesion and function, in terms of P-450 monooxygenase activity and albumin synthesis.[85] Gomez et al. (1997) have used hydrogel hollow fibers as a vehicle to transplant hepatocytes into the peritoneum of Gunn rats and demonstrated bilirubin conjugation in subjects.[57] Chitosan, the deacetylated form of the structural polymer chitin, has been successfully used to construct living and functional hepatocyte organoids from rat and fetal porcine hepatocytes.[68,80]

25.5.2 Cell–Cell Interactions and Topography

25.5.2.1 Cell–Cell Interactions

Interactions between the parenchymal and nonparenchymal cells are of fundamental importance in modulating cell growth, migration, and differentiation. These interactions are directly related to a variety of biological phenomena, such as those observed during development,[95] cancer,[96] pathophysiology,[97] wound healing,[98] and tissue engineering.[99,100] It is thought that, heterotypic interactions mediate the liver formation from the endodermal foregut and mesenchymal vascular structures[101] and stabilize the liver-specific functions of the isolated hepatocytes *in vitro*.[102,103] Thus, many heterotypic interactions occur in the adult liver that coordinate the organ function.

25.5.2.2 Coculture Models

The signals exchanged between cells of various origins simulate the *in vivo* intertissular communications. Analyses of such interactions require tedious and rigorously controlled *in vivo* experiments. Numerous coculture and cotransplantation models based on hepatocytes and other types of cells have been developed to study these interactions (Figure 25.3).

These coculture systems may represent a promising tool for fundamental research, such as analyses of drug metabolism, intercellular regulations, and metabolic pathways, as well as for the establishment of hepatocyte tissue banks; thus, may help to develop functional engineered liver organoids. Albumin secretion, cytochrome P-450 activity, glutathione S-transferase, pyruvate kinase, and UDP-glucuronyl transferase activities, tight junctions (ZO-1 detection), gap junctions (connexin 32 detection), and DNA synthesis are the hepatocyte functions tested in most of the coculture systems (Table 25.3). It is most likely that the liver-specific functions of hepatocyte cocultures are induced by freely secreted cytokine signaling or cell-associated signaling from insoluble extracellular matrix or membrane-bound proteins.[104–106]

Hepatocyte and pancreatic islet cocultures have been successfully transplanted using polymeric matrices as an alternative noninvasive approach to hepatotrophic stimulation.[107] Research showed that the alpha cells migrate into the islet-surrounding hepatocytes, whereas beta cells remain immobile. The recipient glucose metabolism was not interfered, and the transplanted hepatocytes did not show any hyperproliferative premalignant foci. Naughton et al. (1994)[108] transplanted cocultures of hepatocytes and liver stromal cells or bone marrow cells on three-dimensional polymer constructs to rats. The findings showed an association with the stroma and extracellular matrix, and hepatocyte growth in a stereotypic manner, with the generation of hepatic structures such as sinusoids. Matsusaka et al.[109] compared the growth of hepatocytes and biliary epithelial cells between spleens transplanted with oval-cell-free and oval-cell-enriched rat liver cells. Intrasplenic transplantation of oval-cell-enriched liver cells showed better hepatocyte growth compared to oval-cell-free liver cells; however, this was not the case with biliary epithelial cells. Canaple et al.

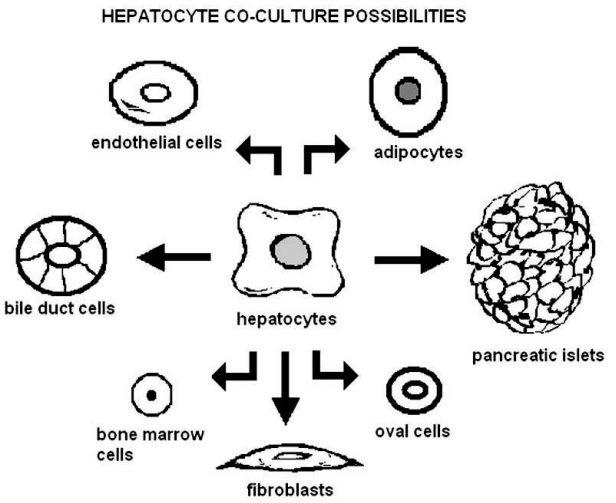

FIGURE 25.3 Coculture possibilities with hepatocytes include the endothelial cells, nonparenchymal cells, bone marrow cells, fibroblasts, pancreatic islets, oval cells, and adipocytes.

TABLE 25.3
Types of Cells Used in Hepatocyte Coculture Systems

Cell Types	Ref.
Endothelial cells	111, 113, 114
Nonparenchymal cells	105, 106, 112–114
Bone marrow cells	108
Fibroblasts	121
Pancreatic islets	107
Oval cells	109
Adipocytes	110

(2001)[110] developed a murine hepatocyte–adipocyte coculture model in biocompatible polyelectrolyte capsules. In their study, encapsulated hepatocytes retained their specific functions, in terms of transaminase activity, and urea and protein synthesis for extended periods.

Cocultures have also been used in bioartificial liver support systems to positively influence the hepatocellular function. Gerlach et al. (1995) have developed a capillary-based reactor with an intraluminal endothelial cell compartment and extraluminal hepatocyte seeding, separated by a semipermeable membrane.[111] Koike et al. (1996)[112] have attempted to cocultivate hepatocytes with nonparencyhmal cells in a perfused multiplate reactor that allows heterotypic contact. Other groups have used cocultures of bovine endothelial cells or rat nonparenchymal cells in conjunction with rat hepatocytes in perfused array of 3-D synthetic biodegradable polymer scaffolds.[113,114]

25.5.2.3 Hepatocyte Spheroids and Dynamic Cell Seeding

Hepatocyte spheroids are aggregates of hepatocytes which are known to stabilize hepatocyte function. They can be manufactured by agitation and aggregation of suspended cells on nonadhesive

substrates and flow perfusion bioreactors under precisely controlled environmental conditions.[115] Hepatocyte spheroids show liver-like morphology and preserve specific metabolic function, in terms of high rate of albumin synthesis and some xenobiotic activity *in vitro*.[116] Yamazaki et al. (1994)[117] have developed a method to manufacture spheroids of same size (i.e., around 350 μm diameter) using patterned thermoresponsible polymers. Ito et al. (1997)[118] have used this approach to obtain 100-μm-diameter cell spheroids. Cell survival, seeding efficiency, and culture conditions (e.g., oxygen uptake) of hepatocyte spheroids may also be improved by forming hybrid hepatocyte spheroids on degradable polymer scaffolds.[119]

25.5.2.4 Topographical Control and Microtemplating

Surface patterning is a functional tool to obtain surfaces with well-known topography. Synthetic polymers are desirable materials, since they permit a more direct pattern transfer technique. Major microtemplating techniques include casting and laser ablation.[120] In a bioartificial liver device, coculture configurations should maximize heterotypic interactions and allow a large proportion of hepatocytes to remain within three to four cell widths from the heterotypic interface. Advances in microfabrication techniques now allow researchers to study the role of the heterotypic interface and the ratio of cell populations. Thus, to engineer a functional tissue equivalent, it seems necessary to construct a topographically-controlled 3-D biomaterial scaffold in larger scale, instead of monolayers. To achieve this goal, the mechanism of the migration of cells inside the 3-D structures should be well understood. The ability to modulate the function of multicellular systems by manipulation of the spatial relationship between cell populations will facilitate more effective *in vitro* reconstruction of liver. Bhatia et al. (1998)[121] have performed studies to understand the role of homo-/heterotypic cell interactions in hepatocyte cocultures toward design of a multiplate micropatterned bioreactor with rat hepatocytes and murine fibroblast cell line, 3T3-J2. In their study, it was found that the cell ratio of the designed bioreactor was a major criterion to obtain maximal hepatocellular function.

25.6 ANGIOGENESIS

The mass transport seems to be one of the key elements in constructing the liver organoid that is highly metabolic (and large, > 200 μm thick).[122] It is crucial to provide the adequate nutrients and oxygen to — and permit the secretion of wastes, metabolic intermediates, and other products from — the newly forming tissue. These transport processes can proceed only if a vascular network is formed within the organoid. Angiogenesis is the cellular assembly that mediates the growing of new blood vessels from a preexisting microvascular bed, an event controlled by autocrine and paracrine signals, in some natural and pathologic conditions.[123] Vascular-affecting (angiogenic) growth factors and some extracellular proteins with receptors responsive to endothelial cells are the principal mediators of angiogenesis. Findings support the possibility of stimulating localized neovascularization, by site-specific delivery of angiogenic growth factors (e.g., vascular endothelial growth factor, endothelial cell growth factor) from degradable or nondegradable polymer devices (e.g., microspheres, scaffolds).[124,125] By using this approach, functional liver organoids made up of fetal porcine hepatocytes in chitosan scaffold have been successfully transplanted to rats.[68] Recently, this approach has been applied to pancreatic islet transplantation in rats using scaffolds derived from both synthetic polyesters and polysaccharides.[126] Endothelial cell-hepatocyte cocultures had previously been used in several BAL designs to maintain the heterotypic contact.[111,113,114] The use of endothelial progenitors for the neovascularization of scaffolds may have implications for liver tissue engineering. Another approach of vascularizing liver organoids is the use of hepatocytes that have been transfected with the gene of an angiogenic factor.[127]

25.7 FUTURE PROSPECTS

The emerging field of tissue engineering involves a multidisciplinary effort, merging the fields of cell and molecular biology, materials science, and surgical reconstruction to engineer new tissue. To date, a great number of tissue engineering scaffolds modified with cell-adhesion peptides, proteins, and growth factors have been tested to engineer liver constructs. Now the focus has shifted toward the three-dimensional microfabrication in larger scale. However, techniques such as coculturing of hepatocytes with parenchymal and other cell types, incorporation of neovascularization vehicles, and the use of dynamic cell seeding (bioreactors) still need to be further evaluated.

Liver tissue engineering will eventually benefit from the emerging science of stem cell biology and signal transduction. Concerning embryonic stem cell research, there are several obstacles (scientific and ethical) that need to be overcome before ES cells can be a reliable source for tissue engineering. Results from hepatic stem cell research underline the need for further investigations, to fully characterize these cells under both *in vitro* and *in vivo* conditions. Thus, most of the current *ex vivo* culture and expansion protocols are for the mixed liver cell populations at distinct maturational stages. Finally, the novel approach using the acellular biomaterial scaffold component to mobilize the stem/progenitor cell reservoirs (hepatic or hematopoietic) of the body presents potential for the engineering of functional liver tissue.

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26 Smart Biodegradable Hydrogels with Applications in Drug Delivery and Tissue Engineering

Carlos Elvira, Gustavo A. Abraham, Alberto Gallardo, and Julio San Román

CONTENTS

26.1 INTRODUCTION

Hydrogels are hydrophilic three-dimensional networks that are crosslinked by chemical or physical bonds and swollen by water.[1] It is well known that they have applications as biomaterials because of their permeability to small molecules, soft consistency, low interfacial tension, facility of purification, and mainly high equilibrium water content, which make their physical properties

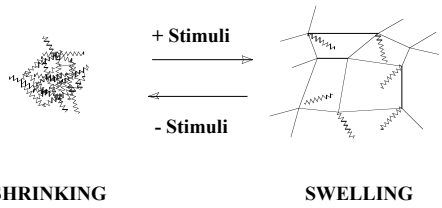

FIGURE 26.1 Behavior of stimuli-responsive hydrogels.

similar to those of living tissues.[2,3] Hydrogels can be applied as artificial skin,[4] as contact lenses,[5] as an interface between bone and an implant,[6] as blood contact materials,[7] and in controlled-release applications for the delivery of bioactive compounds such as enzymes, contraceptives, anticoagulants, etc.[8] Stimuli-responsive or smart hydrogels are polymers that respond with dramatic property changes to small changes in the environment. These polymers undergo fast changes in microstructure from a hydrophobic to a hydrophilic state (sol to gel, or collapsed to expanded morphology), which are triggered by environmental changes and are observed at macroscopic level as in size or swelling degree.[9] These changes can be also reversible as the systems return to their initial state when the stimulus is removed. The common stimuli applied are changes in pH or temperature, which induce neutralization of oppositely charged groups or changes in the efficiency of hydrogen bonding, respectively, which can expand or collapse the corresponding hydrogels as is shown in Figure 26.1.

Recent advances in the design of stimuli-responsive polymers have created opportunities for novel biomedical applications. Stimuli-responsive changes in shape, surface characteristics, solubility, formation of an intricate molecular self-assembly, and sol-gel transition have enabled several new applications in the delivery of therapeutics, tissue engineering, cell culture, bioseparations, and sensor actuator systems.[10] In this sense, it has to be pointed out that hydrogels with applications in tissue engineering and drug delivery are usually proven in both fields due to their intimate ties to biomaterials. This chapter describes different stimuli-responsive hydrogels attending to the type of stimuli applied: temperature, pH, glucose concentration, and others such as electric fields, magnetic field, and ultrasound. In addition, new systems are described as the applications of all of them in drug delivery and tissue engineering, paying special attention to biodegradable or partially biodegradable systems.

26.2 TEMPERATURE-SENSITIVE HYDROGELS

Temperature-sensitive (T-sensitive) hydrogels are among the most commonly studied class of environmentally sensitive polymer systems in drug delivery research.[11,12] In order to have a hydrogel that dramatically changes its swelling degree in water, at least one component of the polymer system should possess temperature-dependent solubility in water. The hydrogel constituents must be insoluble above or below a certain temperature, called lower or upper critical solution temperature (LCST or UCST, respectively). For drug release applications, the results of LCST systems are much more interesting.

Most polymers increase their water solubility as the temperature increases. Hydrogels composed of LCST polymers shrink as the temperature increases above the LCST. This type of swelling behavior is known as inverse (or negative) temperature dependence. The inverse temperature-dependent hydrogels are made of polymer chains that either possess moderately hydrophobic groups (if too hydrophobic, the polymer chains would not dissolve in water) or contain a mixture of hydrophilic and hydrophobic segments. At low temperature, hydrogen bonding between hydrophilic segments of the polymer chain and water molecules are the dominant interactions, leading to enhanced dissolution in water. However, as the temperature increases, hydrophobic interactions

TABLE 26.1
Polymers Showing LCST in Water

Polymer	LCST (°C)
Poly(*N*-isopropylacrylamide), PNIPAAm	~32
Poly(vinyl methyl ether), PVME	~40
Poly(ethylene glycol), PEG	~120
Poly(propylene glycol), PPG	~50
Poly(methacrylic acid), PMAA	~75
Poly(vinyl alcohol), PVA	~125
Poly(vinyl methyl oxazolidone), PVMO	~65
Poly(vinyl pyrrolidone), PVP	~160
Poly(silamine)	~37
Methylcellulose, MC	~80
Hydroxypropylcellulose, HPC	~55
Polyphosphazene derivatives	33–10
Poly(*N*-vinylcaprolactam)	~30
Poly(siloxyethylene glycol)	~10–60

among hydrophobic segments become strengthened, while hydrogen bonding becomes weaker. The final result is the shrinking of the hydrogels due to inter-polymer chain association through hydrophobic interactions. Many polymers and copolymers exhibit temperature-responsive phase transition behavior. Table 26.1 shows some examples of polymers showing LCST in water.

The rule of thumb is that the introduction of a more or less hydrophobic component would either lower or increase the LCST of the final polymer. In general, as the polymer chain contains more hydrophobic constituent, the LCST becomes lower. The LCST can be changed by adjusting the ratio of hydrophilic and hydrophobic segments of the polymer, by modifying the chemical structure of the monomer side chain or by making amphiphilic copolymers with hydrophobic and hydrophilic monomers.[13] Figure 26.2 shows the chemical structures of some of the T-sensitive polymers.

The area of T-sensitive gels has been dominated by poly(*N*-alkylacrylamides), poly(*N*-isopropylacrylamide) (PNIPAAm) being probably the most extensively investigated. PNIPAAm shows a very well-defined LCST at about 32°C, it means that this polymer is soluble in water below 32°C.

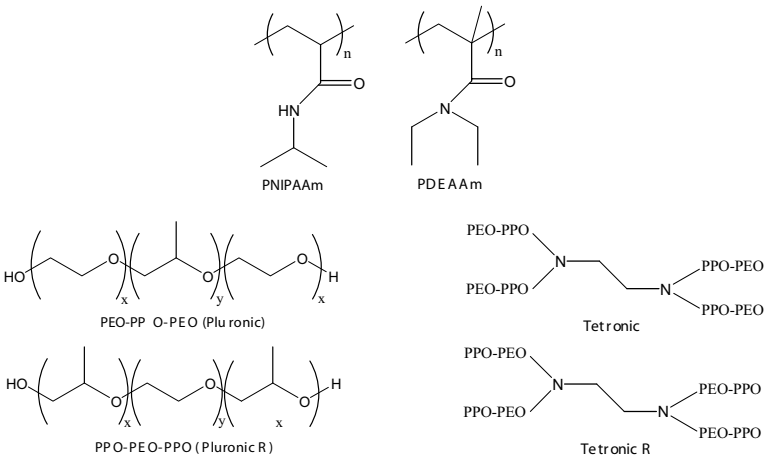

FIGURE 26.2 Chemical structure of some T-sensitive polymers.

Copolymerization of NIPAAm with more or less hydrophilic monomers to alter the LCST may result in hydrogels with more versatile properties, such as faster rates of shrinking when heated through the LCST or sensitivity to additional stimuli. Poly(*N,N*-diethylacrylamide) is also widely used as gel constituent because its LCST is in the range of 25–32°C, also close to the body temperature.

If the polymer chains in hydrogels are not covalently crosslinked, T-sensitive hydrogels may undergo sol-to-gel phase transitions, instead of swelling–shrinking transitions. The thermally reversible gels (TRG) with inverse temperature dependence become sol at lower temperatures. TRG gels are mainly represented by some natural occurring polymers such as gelatin (protein obtained from the collagen hydrolysis), polysaccharides such as agarose, amylopectin, amylase, and Gellan® (composed of glucose and -*D*-glucuronic acid and α-*L*-rhamnose). These polymers are sol at high temperatures and become gel at lower temperatures by formation of aggregation of double helices that act as knots.[14] Cellulose derivatives follow the TRG (not inverse) behavior, which is basically affected by the substitutions of the hydroxyl group. Block copolymers composed of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) display also an inverse T-sensitive property.[15] Because their LCST is around the body temperature, they have been used widely in the development of controlled drug delivery systems and injectable gels for tissue engineering, based on the sol-gel phase conversion at the body temperature. Block copolymers based on PEO–PPO sequences are a family of commercially available triblock copolymers which have the following trade names: Pluronics® and Pluronics® R (BASF Corporation), or Poloxamer (ICI), Tetronics®, and Tetronics® R (BASF Corporation). PEO-PLGA poly(lactic/glycolic acid)-PEO triblock copolymers show sol-gel transitions in aqueous solutions at 30°C, resulting in the formation of an *in situ* transparent gel with maintained structural integrity and mechanical strength.[16] Similar systems based on aqueous solutions of PEO-g-PLGA and PLGA-g-PEO form at 37°C soft gels that are biodegradable and can be applied in tissue engineering.

Beyond traditional copolymeric gel systems, T-sensitive gels can be designed from a blend of two interpenetrating polymer networks (IPNs), where one crosslinked network is intertwined with another. New designs of T-sensitive hydrogels involve also the use of T-sensitive crosslinking agents.

26.2.1 "On–Off" and Pulsatile Delivery Concepts

Figure 26.3 shows different modes of the controlled release of drugs, and of proteins in particular, from T-sensitive hydrogels. When a hydrophilic drug is incorporated into T-sensitive gel, it can show a Fickian release below the LCST, the details of which depend on the swelling degree of the gel and the tortuosity of the pathway the drug must take (Figure 26.3A). Conversely, a more hydrophobic drug can show Fickian diffusion from the collapsed gel above (Figure 26.3B). If a drug is loaded below the LCST, it can be squeezed out above the LCST due to the pressure generated during gel collapse (Figure 26.3B). A similar idea was realized with gels immobilized within porous membranes. Expanded polymer chains (below LCST) closed pores, limiting the diffusion of drugs, while shrunken polymer chains (above LCST) open its pores, allowing high diffusion of drugs. This behavior is the so-called molecular gates (Figure 26.3C). Diffusion controlled systems can be also achieved with a heterogeneous gel formed by a dense "skin" layer of the collapsed or precipitated polymer layer while the core remains swollen (Figure 26.3D). The skin barrier is formed upon a sudden temperature change due to the faster collapse of the gel surface than the interior. T-sensitive hydrogels can also be placed inside a rigid capsule containing holes or apertures. This kind of gel structure allowed the modeling of an auto-feedback glucose-insulin system for the insulin pulsatile drug release.[16] Thermosensitivity may allow the control of enzymatic activity. This involves the concept of immobilization of either the enzyme or substrate in the swollen gel, resulting in turning the enzymatic reaction on and off below and above LCST due to diffusion limitations in the collapsed, dense polymeric phase. Thus, the closing and opening of the pathways for molecular diffusion can be achieved by reswelling and deswelling the pores as the temperature is raised and then lowered around LCST.

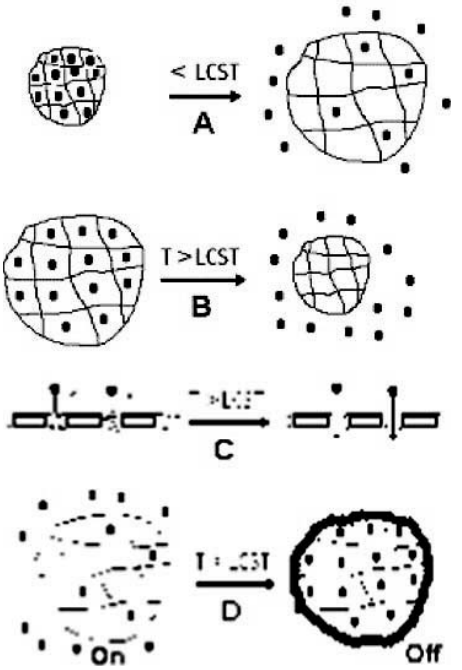

FIGURE 26.3 Modes of drug delivery from T-sensitive hydrogels. (From Bromberg, L.E. and Ron, E.S., *Adv. Drug Deliv. Rev.,* 31, 197, 1998. With permission.)

26.2.2 Applications of T-Sensitive Hydrogels

T-sensitive hydrogels have been extensively reviewed due to their unique properties and applications.[17–20] Hydrogels are classified into (1) negatively thermosensitive, (2) positively thermosensitive, and (3) thermally reversible gels. The water swelling of most hydrogels is influenced by temperature in terms of sensitivity and dependency: an increase (positive thermosensitivity) or a decrease (negative thermosensitivity) of swelling with increasing temperature. The third class is the reversible gel, in which the network connections are reversible; they will always be opening, closing, and interchanging.

26.2.2.1 Negatively T-Sensitive Drug Release Systems

Negatively T-sensitive hydrogels can be secured by placing them inside a rigid matrix or by grafting them to the surface of rigid membranes. For example, a composite membrane was prepared by dispersing PNIPAAm hydrogel microparticles into a crosslinked gelatin matrix[21] or by encapsulating the drug core with ethylcellulose containing nano-sized PNIPAAm hydrogel particles.[22] For making stable thermally controlled on-off devices, PNIPAAm hydrogel can be grafted onto the entire surface of a rigid porous polymer membrane.[23]

Clinical applications of NIPAAm-based T-sensitive hydrogels have limitations since they are not biodegradable but have been evaluated as drug release carriers consisting on semi-IPN of PNIPAAm and poly(tetramethylene glycol) crosslinked with *bis*-acrylamide (BMA), analyzing the release of indomethacin as model drug, showing an on-off pulsatile behavior and a squeezing release mechanism.[24] Recently, a newly designed hydrogel with both T-sensitivity and biodegradability based on PNIPAAm crosslinked with degradable poly(amino acid) was studied.[25] T-dependent enzymatic degradation of semi-IPN hydrogels consisting of dextran grafted with T-responsive chains (LCST) and a T-responsive crosslinked matrix (UCST) was also investigated. Only between both temperatures, enzymatic degradation proceeded. The designed semi-IPN hydrogel is therefore advantageous to achieve enzymatic degradation at a specific temperature range.[26] Poly(NIPAAm-

co-AA [acrylic acid]) gels have been applied as extracellular matrix for pancreatic islets in biohybrid pancreas[27] and as reversible 3-D matrix for the culture of articular chondrocytes that proliferate adequately on the matrix that is then removed by temperature lowering.[28]

Two T-sensitive acrylic polymers, poly(2-ethyl-2-pyrrolidone-methacrylate) (PEPM) and poly(2-ethyl-2-pyrrolidine-methacrylate) (PEPyM), have been prepared in our laboratory showing LCST of 34 and 15°C, respectively.[29] Both systems were crosslinked with *N, N*-methylenebisacrylamide (BMA) and their swelling degrees were found to be sensitive to the temperature. Polymers were charged with the drugs diflunisal and ketoprofen and the release profiles were found to be dependent on the temperature following a hydrogel squeezing mechanism.[30]

26.2.2.2 Positively T-Sensitive Drug Release Systems

Certain hydrogels formed by IPNs show positive thermosensitivity, i.e., swelling at high temperature and shrinking at low temperature. IPNs of poly(acrylic acid) and polyacrylamide (PAAm) or P (AAm-co-BMA) display this behavior.[31] The swelling of these hydrogels was reversible, responding to stepwise temperature changes. This resulted in reversible changes in the release rate of a model drug, ketoprofen, from a monolithic device.

26.2.2.3 Thermoreversible Gels

The vast majority of the drug delivery TRG systems use pluronics (or poloxamers) and tetronics.[11,32] Some of them has been approved by FDA and EPA for applications in food additives, pharmaceutical ingredients, and agricultural products. For parenteral application of thermoreversible gels, it is most desirable that they are biodegradable. To provide biodegradable capacity, the PPO segment of PEO–PPO–PEO block copolymers is often replaced by a biodegradable poly(*L*-lactic acid) segment. The molecular architecture was not limited to the A–B–A block copolymer, but expanded into three-dimensional, hyperbranched structures, such as a star-shaped structure. Proper combinations of molecular weight and polymer architecture resulted in gels with different LCST values.[33]

Pluronic F127 at 20 wt% in aqueous solutions has been applied in burn treatment and other wound-healing applications.[34] More recently, PEO–PPO–PEO copolymers have been used in cartilage regeneration layers on the host bone, by preparing a chondrocyte-polymer solution that solidifies after a few minutes of application and can be applied as injectable cartilage formulation, showing excellent promising results.[35] PEO gels have been also tested as alternative synthetic polymers as injectable 3-D matrix for the delivery of isolated chondrocytes. In these applications, and in order to obtain bioerodible matrices, PEO was linked to photopolymerizable acrylate groups, which allow the gel to be transformed from liquid to solid by polymerization reaction using UV light.[36] Polymer/cell suspensions were injected to different mouse locations and cartilage formation was observed by histological tests.

26.3 pH-SENSITIVE HYDROGELS

26.3.1 Basic Concepts

Polymers with ionizable groups, known as polyelectrolytes, such as polycarboxylic acids or polyamines, are affected by pH changes in terms of the degree of ionizations, which influences the solubility or conformation of the polyelectrolyte. When these polymers are crosslinked, the swelling degree is a function of pH and other factors such as ionic strength.[37] The pH-sensitivity of polyelectrolyte hydrogels is influenced by the nature of the ionizable groups, the crosslinking density, the polymer composition, and the hydrophobicity of the polymer backbone. The nature of the functional groups affects the pH-sensitivity in terms of the swelling degree, depending on the basic or acidic character of the chemical groups present in the macromolecules. The crosslinking density affects the solute permeability in terms of bioactive compounds release in several applica-

tions; the higher the crosslinking density, the lower the permeability, especially significant in the case of high-molecular-weight solutes. The hydrophobicity of these polymers can be controlled by copolymerizing the hydrophilic ionizable monomers with hydrophobic monomers with or without pH-sensitivity moieties. The overall permeability of the hydrogels decreased when increasing the polymer hydrophobicity, increasing its dependency on the pH.

The pH range of fluids in various segments of the gastrointestinal tract, from pH 2 in the stomach to pH 8 in the mid and left colon, may provide environmental stimuli for responsive drug release. The pH in the human plasma is around 7.4; however, the tumor pH in mammary carcinoma is somewhat lower. Taking into consideration this pH change in the human body, several groups,[38–40] starting in the 1980s, have performed studies on polymers containing weakly acidic or basic groups in the polymer backbone. The charge density of the polymers depends on pH and ionic composition of the solution into which the polymer is exposed. Altering the pH of the solution will cause swelling or deswelling of the polymer. Polyacidic polymers will be unswollen at low pH, since the acidic groups will be protonated and unionized. When increasing the pH, a polynegatively charged polymer will swell. The opposite behavior is found in polybasic polymers, since the ionization of the basic groups will increase with decreasing the pH. Thus, drug release from reservoir or matrix devices made from these types of polymers will display rates that are pH dependent.

Figure 26.4 exhibits the chemical structure of typical examples of pH-sensitive polymers with anionic groups such as poly(acrylic acid) (PAA), poly(methacrylic acid) (PMA), and poly(ethylacrylic acid) (PEAA) and with cationic groups such as poly(dimethyl aminoethyl methacrylamide) (PDAEM), poly(lysine) (PL), poly(ethylene imine) (PEI), chitosan, hyaluronic acid, and alginate gels, among others. Hydrogels made from PAA and derivatives are the most applied in this type of sensitive polymers.

Pulsatile pH sensitivity (see Figure 26.5) is an important factor when considering these types of hydrogels to be applied as drug delivery carriers. It is also important to consider a pH transition of the pH-sensitive hydrogels near the physiological pH as shown by novel synthetic hydrogels with ionizable groups such as sulfonamide-containing polymers.[41]

Chitosan, (1,4)-linked 2-amino-2-deoxy-*D*-glucan, is a biodegradable and biocompatible polymer that in solution exhibits a liquid-gel transition about pH 7 when pH changes from acidic to neutral. It is a cationic polysaccharide obtained from the deacetylation of chitin, whose molecular weight and degree of deacetylation causes chitosan to have different properties, and has been extensively applied in the biomedical field.[42] Hyaluronic acid exhibits a similar chemical structure, a glucosaminoglycan with a carboxylic group, and also has been applied in several tissue engineering applications, which hydrogels are formed by esterification with hydrazide derivatives obtaining covalent crosslinked gels.[43] Another biodegradable polysaccharide that presents pH sensitivity is the alginate gel composed by ionically crosslinked mannuronic acid and glucuronic acid repeating units, which in solution form gels with divalent cations such as calcium.

26.3.2 Applications of pH-Sensitive Hydrogels

Most of the crosslinked hydrogels with pH-sensitivity are not biodegradable. However, some attempts have been performed combining biodegradable polymers with ionic hydrogels or by incorporating biodegradable groups to the polymers backbone, in applications as drug delivery carriers or in tissue regeneration processes.

Poly(propylacrylic acid) (PPAA) has been applied as a pH-sensitive membrane-disruptive system to enhance the release of drugs from the acidic endosomal compartment to the cytoplasm, the *in vitro* transfections in cell culture, and improve the *in vivo* wound healing.[44] Microparticles of copolymers of methacrylic acid with grafted polyethylene glycol have been evaluated for the nasal administration of budesoine (drug used in the treatment of allergic, seasonal rhinitis and asthma) by using a drug load of 25 wt%, showing that by its mucoadhesiveness, the gel swelling modulation and pH-sensitivity, make this system a good candidate to be applied in drug controlled

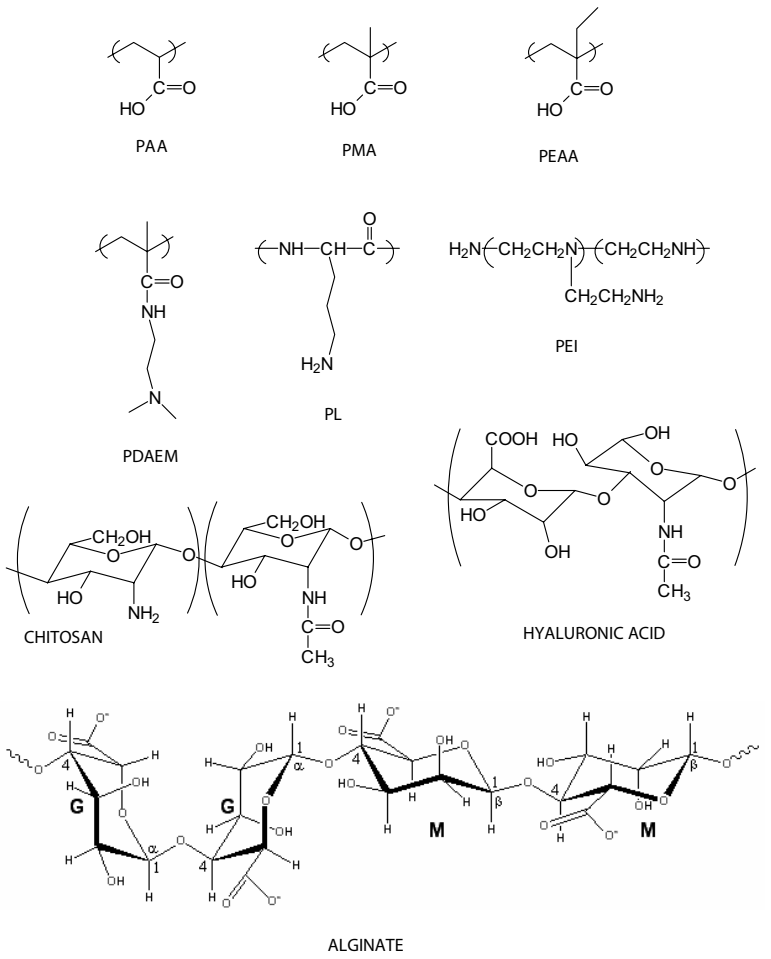

FIGURE 26.4 Chemical structure of some pH-sensitive polymers.

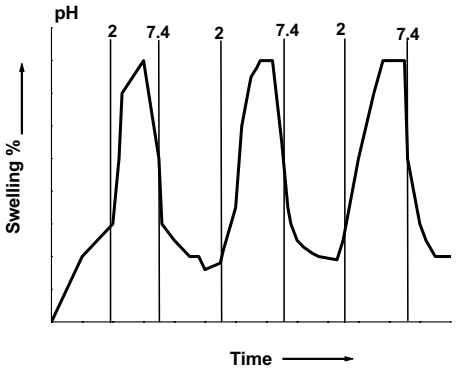

FIGURE 26.5 Pulsatile behavior of anionic pH-sensitive hydrogels in terms of swelling degree when a repetitive pH variation is applied in the range of 2 and physiological pH 7.4.

release at the surface of nasal mucosa *in vivo*.[45] Other type of pH-sensitive polymers applied in drug delivery corresponds to polymeric prodrugs, in which the drug is covalently bonded to the macromolecular chain. This is the case of polymers such as poly(*N*-methacryloylaminoethyl 5-amino salicylamide) or poly(methacryloylethoxyethyl 5-amino salicylic acid) that have been evaluated for the release at different pH of 5-amino salicylic acid exhibiting profiles that indicated that the hydrolytical behavior of the polymers strongly depends on their swelling degree, type of comonomer used, and the nature of the hydrolyzable bond, indicating that they can be applied for colon targeting.[46] Another interesting pH-sensitive polymer is poly(silamine), the most remarkable characteristic of which is that the gel hardened on swelling by formation of rigid molecular locks through ionic interactions.[47] Recently, pH-sensitive polymers have started to be applied in gene delivery and gene therapy research by means of transporting DNA into cells by using cationic polymers such as poly(*L*-lysine), poly(ethylene imine) and amine-containing dendrimers,[48,49] poly(propyl acrylic acid), and poly(ethacrylic acid).[50]

A natural polymer with pH sensitivity is chitosan, which contains free amino groups in its structure and can be protonated in acidic solutions. Graft copolymers of chitosan with *D,L*-lactic acid[51] and with poly(dimethylsiloxane)[52] have been prepared obtaining biodegradable hydrogels that exhibit pH-reversible sensitivity under pH abrupt changes between 7.4 and 2.2, which have potential applications as drug delivery carriers. It has been also applied in tissue engineering in osteoblast differentiation and bone formation. By mixing chitosan with calcium phosphate, injectable composites have been developed that at pH 6.5 behave as a moldable chitosan-ceramic suspension which at physiological pH undergo a phase transition, becoming in entrapment of the ceramic component within the reversible gel matrix.[53] These composites can be applied as bone fillers whose rheological properties can be optimized for injectability, their porosity is suitable for cell adhesion and proliferation, their degradation behavior is slow and can be regulated, and therapeutic drugs can be also incorporated for their release.

Systems consisting in alginate and PNIPAAm semi-IPN hydrogels prepared by radical polymerization of NIPAAm in the presence of alginate, showed pH and T-sensitivity with a maximum swelling degree at pH 4 and a LCST of 32°C, with pulsatile behavior.[54] Alginate gels have been evaluated in mice for cartilage regeneration by slow polymerization of calcium alginate as injectable vehicles of isolated chondrocytes giving excellent results in terms of the solidification of the injected polymer-chondrocytes, which allow the cartilage reconstruction by using a minimally invasive technique.[55] These gels were also evaluated for the treatment of vesicular reflux, a common disease in the pediatric population, by injection of alginate-chondrocytes solution for the cell delivery, showing results that make this system a potential useful treatment.[56] Hyaluronic gels have been applied as injectable material for correcting contour defects in facial skin such as lip augmentation and recontouring,[57] for cosmetic intradermal implants,[58] and as gel matrix of sodium hyaluronate for the delivery of basic fibroblast growth factor in the repair of bone fractures.[59]

Semi-IPN of starch blends with acrylic copolymers of acrylic acid with acrylamide have also shown pH sensitivity with maximum swelling degree at physiological pH and with a Fickian case II swelling kinetics, which corresponds to the most desirable kinetic behavior for a swelling controlled-release material, being also partially biodegradable.[60] Other types of biodegradable hydrogels with pH sensitivity were prepared by Kopecek et al.[61] containing azoaromatic moieties synthesized from polymeric precursors by crosslinking reaction or by a polymer–polymer reaction. The hydrogels degradation was found to be dependent on the synthetic method (giving different crosslinking densities), and on the pH, having valuable applications for oral colon-specific drug delivery. Biodegradable hydrogels based on crosslinking poly(*L*-glutamic acid) (PLG) with PEG are pH-sensitive systems in which swelling degree is also affected by ionic strength.[62] These hydrogels also present pulsatile behavior and have been tested for the release of proteins such as lysozyme drug.

Biodegradable cationic hydrogels have been developed for gene delivery and gene therapy based on poly(*L*-lysine) ester analogue and hydroxyl proline polymers. Taking into consideration that gene transfection process takes less than an hour, the hydrogel was found to degrade within

one day in *in vitro* experiments. Another interesting approach for these types of applications was developed by Benns et al.[63] who developed a graft copolymer of poly(*L*-lysine)-*g*-poly(histidine) in which the lysine part confers the cationic character, and histidine the ability to act as endosomal fusion polymer, requirement to maximize the DNA transport to the cell nucleus.

26.4 GLUCOSE RESPONSIVE SYSTEMS

Insulin, a key hormone produced in the pancreas, is secreted in response to excess of glucose in the blood, as it is observed after a meal. Insulin aids the body by helping transfer glucose into the cells where it is oxidized to produce energy, slowing the breakdown of fatty acids and promoting the uptake of amino acids in muscle. Diabetes mellitus (Type I) disables the body's ability to produce insulin, which prevents the body from regulating blood sugar, among other effects. An elevated blood sugar level caused by the lack of insulin is one of the leading causes of blindness in adults and contributes to heart disease, stroke, and amputations.[64] Daily insulin shots are the present treatment for diabetes, but this treatment is quite insufficient for maintaining physiologically normal glucose levels. The release profiles of insulin are highly variable, giving large initial levels of insulin in relation to glucose, which degrade to inadequate levels over time, and are only replenished by another insulin injection.

Therefore, to mimic the physiological secretion of insulin, the most desirable solution is a self-regulating delivery system with glucose-sensing ability.[64] One of the most challenging problems in controlled drug delivery area is the development of these modulated insulin delivery systems. These systems should respond to different glucose levels in the blood, caused by consuming food, and exude the proper amount of insulin to dispose of the glucose properly. Delivery of insulin is different from delivery of other drugs, since insulin has to be delivered in an exact amount at the exact time of need. Many hydrogel systems have been developed for modulating insulin delivery, and all of them have a glucose sensor built into the system.

26.4.1 pH-Sensitive Membrane Systems

Normal blood pH and temperature are around 7.4 and 37°C. An internal insulin delivery system that responds to changes in one or two variables is a difficult challenge. The temperature change would be very minimal (~ 0.5°C) after the consumption of a meal, and that rise could only be attributed to the heat produced during digestion. The pH change should be more pronounced. In a glucose-rich environment, such as the bloodstream after a meal, the oxidation of glucose to gluconic acid catalyzed by glucose oxidase (GluOx) can lower the pH in the blood to approximately 5.8.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GluOx}} \text{Gluconic Acid} + H_2O_2$$

Thus, GluOx is probably the most widely used enzyme in glucose sensing. This enzyme makes it possible to use different types of pH-sensitive hydrogels for modulated insulin delivery. Hydrogel membranes made of polycations lower the pH and lead to hydrogel membrane swelling due to ionization. If the hydrogel membranes are made of polyanions, self-regulated insulin release is controlled by different mechanisms. A first example is a glucose-sensitive (G-sensitive) hydraulic flow controller that can be designed using a porous membrane system consisting of a porous filter grafted with polyanions and immobilized GluOx. The chains expanded at pH 7 due to electrostatic repulsion among the charges on the polymer chains and collapsed after gluconic acid formation due to the protonation of the carboxyl groups. Thus, the pores are open for the diffusion of insulin. In a second example, insulin can be loaded inside a hydrogel matrix which can be collapsed as a result of lowering the pH. In this case, insulin release is enhanced due to the "squeezing" action

of the collapsing hydrogel. A third example is a system where a GluOx-containing hydrogel covers a pH-sensitive erodible polymer that contains insulin, the polymer erosion, and thus insulin release, is controlled by the lowering of the local pH.

A purely pH-responsive hydrogel with T-sensitivity does have promise as an insulin delivery mechanism. By regulating certain factors, the candidates can be designed to swell and deswell at a specified pH. The next logical step would be a G-sensitive hydrogel, which volumes change capacity for releasing insulin when the conversion reaction takes place.

Other hydrogels entrap GluOx within the membrane. Diethylaminoethyl methacrylate-co-PEG monomethacrylate is used and the dynamic swelling behavior and varying pH levels were investigated. While the swelling response was faster than the deswelling, the hydrogel still exhibits a start/stop mechanism, which is crucial for insulin delivery.[65]

Systems based on concanavalin A (Con A) are very interesting and show specific behavior with potential applications. Con A is a four-site glucose-binding protein obtained from the jack bean plant, *Canavalia ensiformis*. In this system, insulin molecules are attached to a support or carrier through specific interactions which can be interrupted by glucose itself. Con A solutions exhibit a sol to gel transition in the presence of free glucose, and its ability to release insulin was investigated. Con A does provides an on/off mechanism, which is needed in physiological insulin delivery, but a major drawback is that it can cause an immune response if used *in vivo*.[66]

PEG hydrogels crosslinked with dextran were charged with insulin into the PEG phase where the concentration decreased with increasing molecular weight of PEG. Drug release from the hydrogel occurred with the degradation of dextran and the diffusion of insulin through the system. It is noted that with higher crosslinked polymers, the degradation rate, and hence insulin release, is slowed, which opens the door for designable hydrogels with specified release times. Though the degradation time of the hydrogels was controlled by the dextranase concentration used, even with a fairly high concentration, the time needed for insulin release was on the order of hours.[67]

26.5 OTHER TYPES OF SENSITIVE HYDROGELS

26.5.1 Electric-, Magnetic-, and Ultrasound-Responsive Hydrogels

26.5.1.1 Electric-Responsive

These types of hydrogels, mainly ionizable polyelectrolytes, are sensitive to electric stimuli in terms of swelling or deswelling and can be applied in the release of bioactive compounds following a pulsatile behavior.[68] Systems based on copolymers of poly(2-acrylamide-2-methylpropanesulfonic acid-co-butyl methacrylate) have been charged with edrophonium chloride as model drug, and evaluated by applying an on–off voltage showing the complete release of the drug.[69] Other system formed by the combination of cationic poly(allylamine) with anionic bioactive heparin showed an on–off heparin release profile in response to on–off stimuli of electric current, where the initial complex of polymer–heparin is insoluble in water and becomes soluble upon the application of the electric field.[70] Polypyrrole is another conducting polymer that has been applied in nerve cell regeneration processes by applying an electric current through the oxidized form of the polymer in rats as animal model, obtaining enhanced nerve cell regeneration.[71]

26.5.1.2 Magnetic-Responsive

This is other type of stimuli-responsive systems, which basically uses magnetic fields to magnetically control the drug delivery from a polymeric hydrogel in which small magnets have been incorporated. These systems have been tested for the enhanced release of serum albumin[72] or insulin[73] by applying an oscillating magnetic field and have obtained promising results that may be effective in more complete clinical systems.

26.5.1.3 Ultrasonic-Responsive

Ultrasound is used to improve drug permeation through biological barriers as well as to enhance erosion in bioerodible polymers such as polyglycolide, polylactide, and polyanhydrides in drug delivery systems. Concerning hydrogels sensitive to ultrasound, ethylenevinyl alcohol copolymers have been evaluated for the release of 5-fluorouracil and bovine insulin by applying ultrasound (1 MHz), increasing the release rates of the corresponding drugs.[74] Similar phenomena were observed in PEG-grafted PHEMA surfaces by a pulsatile release of insulin along 4–5 days, being a system useful to deliver biological peptide drugs in a pulsatile manner.[75]

26.5.2 Protein-Responsive Hydrogels

26.5.2.1 Enzyme-Sensitive Hydrogels

Some enzymes are applied as signals for diagnosis of physiological changes and for the specific drug delivery in certain organs and can be applied as enzyme sensors and enzyme-sensitive drug delivery systems.[76] These hydrogels are based on biodegradable polymers which degradation mechanism is based on enzymatic attacks. Several works focused on this topic have been developed by Kopecek et al.[46] in the pH-sensitive hydrogels part, in which azoreductase, an enzyme produced by the microbial flora of the colon, degrades the azoaromatic crosslinking bonds of the hydrogels. They are copolymers of *N,N'*-dimethylacrylamide with acrylic acid that protect the charged protein drugs from enzymatic degradation in the stomach because of the low swelling degree at acidic pH, being swollen in the colon where azoreductase degrades the polymer matrix and the protein drugs are delivered. A similar mechanism occurs in the release of drugs from dextran hydrogels where the enzyme dextranase, also present in the colon, degrades *in vitro* the polysaccharide being the drug release controlled by the presence of the enzyme.[77]

26.5.2.2 Antigen-Responsive Hydrogels

Antigen and antibody are associated to the immune responses to protect the organism from infections and bind in specific sites by electrostatic, van der Waals, or hydrophobic interactions or by hydrogen bonds. In this sense, the preparation of sensors for immunoassays and antigen sensing can be performed on the antigen–antibody recognition basis. An interesting system developed by Miyata et al.[78] is an antigen-responsive hydrogel whose preparation strategy is based on the reversible character of the antigen–antibody bond, which is applied as crosslinking points of a hydrogel semi-IPN. In the absence of antigen, the hydrogel shrinks and swells in its presence. First, the antigen and the antibody are separately bonded to the *N*-succinimidylacrylate. The antibody coupled to the monomer was copolymerized to acrylamide (AAm) by using a radical redox initiation system, and then antigen-monomer was copolymerized with AAm and with *N,N*'-methylenebisacrylamide (MBAAm) in the presence of the antibody copolymer to obtain the hydrogel semi-IPN. The hydrogel swelling degree increases in the presence of free antigen as well as the crosslinking density of the semi-IPN, due to the antigen-antibody interactions. Another antigen-responsive hydrogel based on polymerizable antibody Fab fragment has been prepared by Lu et al.[79] by radical copolymerization with NIPAAm, with MBAAm as crosslinking agent using also redox initiator systems. The hydrogel responsiveness depends on the pH, temperature, and on the antibody content and was found to be reversible with a 50 wt% of Fab and at 33.7 and 36.8°C, at physiological pH.

26.6 CONCLUSIONS

The systems discussed in this chapter describe the attempts developed recently in the stimuli-responsive hydrogels field, with applications in drug delivery and tissue engineering. Despite the

extensive research effort during the last 15 years, practical applications of smart hydrogels in drug delivery are still to emerge as commercial products. It has to be pointed out that the discussed hydrogels are still in the early development stage, and further research has to be performed in order for them to become clinical alternative materials. In this sense, some aspects to be considered and investigated are the design of new smart biodegradable hydrogels based on polymer blends or copolymers, as well as the development of new biodegradable synthetic polymers sensitive to external stimuli; the biocompatibility of polymers in terms of toxicity, carcinogenesis, and immunogenicity; the response times of these systems to stimuli, and their ability to provide practical levels of the desired bioactive compound in the mentioned applications.

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27 Skin Tissue Engineering Part I — Review

Dietmar W. Hutmacher, K. W. Ng, and H. L. Khor

CONTENT

27.1 INTRODUCTION

The clinical requirement for artificial graft materials to promote effective wound repair is large. Examples of chronic or seriously deficient wounds include pressure sores diabetic skin ulcers, venous stasis ulcers, burn injury, and defects arising following tumor excision. In the United States alone, there are 100,000 hospital-treated burns per year and 600,000 cases of surgical skin excision — burn wounds costing an estimated $70 million per annum. The age-related problem of nonhealing dermal wounds is far larger, with 11–12 million patients being treated in the United States. This will become a greater problem as the population ages. Up to 15% of diabetic patients develop foot ulcers, leading to 50,000 amputations per year (U.S.). Almost half will die or lose the opposite leg within 3 years. Clearly, the clinical problem of dermal ulceration is huge — and likely to grow in Europe, Asia, and the United States.

Specific wound types can be categorized using several different criteria; for the purpose of this chapter, two broad categories will be used, namely acute and chronic. Acute refers to elective wounds, surgical wounds, and burns. To date, burns alone have been considered as suitable lesions for tissue engineering, this is due to the relative ease with which surgical wounds repair themselves, and potential mortality associated with burns.

The cause of tissue breakdown in most chronic wounds is due to poor nutrition, tissue ischemia, age, or a combination of these factors. Examples of such wounds include venous leg ulcers, diabetic ulcers, arterial ulcers, and pressure sores. Chronic wounds are considered to be on the increase in

FIGURE 27.1 A wide array of wound management products are commercially available for various wound healing applications.

developed Western markets, this is due to their increasing incidence with age, and the demographic shift occurring in these populations.

The general trend from a clinical point of view in skin tissue engineering is to study the efficiency and efficacy on burn wounds first, before application to chronic wounds. The reasons for this are both economic and ethical. Treatment concepts based on tissue-engineered skin grafts often require the combination of expensive culturing procedures, scaffolds, and matrices. Such therapies may initially be used only in life-threatening situations, such as major burns, where conventional wound-care procedures (such as split-skin grafting) may not be possible, the risks and costs presenting less of an issue. In general, however, the burns market may not be large enough to justify development. Therefore, most tissue engineering companies have the market strategy that their products can be used in the burn market as well as chronic wound market.

Chronic wounds are not considered to be life threatening. As a consequence, justification for the use of tissue-engineered products — e.g., in preference over less-expensive wound management products (Figure 27.1) — needs to be based on factors such as improved quality of life and cost-effectiveness. For example, treatment with tissue-engineered products should result in improved healing rates and reduced nursing time. In addition, some tissue-engineered approaches may offer reduced recurrence rates and, in the case of diabetic ulcers, reduced risk of limb loss through amputation.

27.2 SKIN TISSUE ENGINEERING FROM A HEALTH CARE PERSPECTIVE

Tissue engineering is a young and interdisciplinary scientific discipline, but it offers exciting opportunities to improve the quality of health care for today's patients. Lured by its potential in

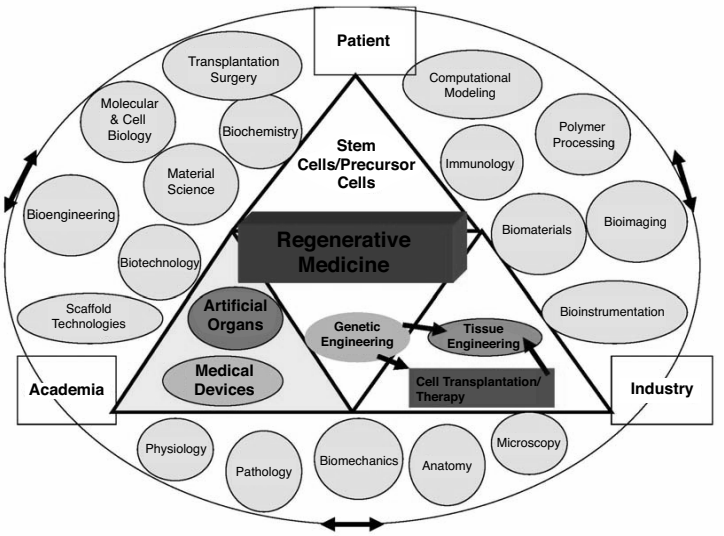

FIGURE 27.2 Tissue engineering is a truly multidisciplinary field, and its success will hinge on close interactions between scientists, engineers, and health care professionals.

the 1990s, several start-up companies, pharmaceutical as well as biotech corporations, and medical device enterprises did invest heavily in this sector. However, most of those companies strive for survival nowadays.[1] When attempting to justify the use of tissue-engineered products in burn and chronic wounds, several important cost-related issues need to be addressed: Does the inevitable initially higher cost associated with tissue-engineered skin grafts, when compared on a unit price basis with conventional therapy, translate into more clinically predictable or faster healing rates as well as improved overall cost-effectiveness? In performing such calculations over the entire treatment period, it is important to consider indirect health care costs, such as duration of hospitalization, nursing time, and complications after treatment, improvement of patient's quality of life, and duration of medical leave. In order to achieve cost-effectiveness, new tissue-engineered skin products will need to perform as complete skin equivalents in burns and chronic wounds and stimulate and accelerate the healing processes. In this respect, the design and fabrication of novel matrices for the epidermal as well as dermal part are a condition *sine qua non*.

From a scientific point of view, tissue engineering can be defined as a truly multidisciplinary field (Figure 27.2) that applies the principles of life science, engineering, and basic science to the development of viable substitutes that restore, maintain, or improve the function of human tissues.[2] Modern isolation and culturing techniques of any type of human cells including skin, muscle, cartilage, bone, endothelial, and mesenchymal stem cells provide the basis for tissue engineering.

Naturally derived or synthetic biomaterials are fashioned into scaffolds that when cultured and implanted in combination with cells provide a template that allows such constructs to form new soft and hard tissues, during which time the scaffold gradually degrades and is finally metabolized. The significance of tissue engineering research in regard to the technological, social, and economical impact has been discussed in several review papers.[1,3,4] Over the last 5 years, leading research institutions in the U.S., Europe, Australia, Japan, and Singapore have embarked on multimillion-dollar initiatives to develop strong national and regional tissue engineering programs.[5]

It was reported that in 2001, tissue engineering research and development was being pursued in the U.S., Europe, and Australia by 3300 scientists and support staff in more than 70 start-up companies or business units. Investment since 1990 exceeded $3.5 billion. The net capital value of the 16 publicly traded tissue engineering start-ups had reached $2.6 billion. Firms focusing on skin, cartilage, and bone comprise the fastest growing market segment.[6]

Skin tissue engineering has been ongoing for at least the past two decades and represents the most successful clinical application of tissue engineering in the present.[7–9] Despite the optimistic clinical reports and numerous commercial activities, the currently available skin tissue engineering concepts are prone to some technical and functional restrictions, which makes it sometimes difficult to predict the final outcome from a clinical point of view. Today, it is broadly accepted that scaffolds specifically designed and fabricated for modern skin tissue engineering might overcome several of the problems, such as fragility, handling, contraction, and, with an optimal preparation of the wound bed, improve the take rate, otherwise not reproducibly predictable before grafting. This review discusses the history and state of the art of skin tissue engineering from a matrix point of view and will attempt to give an outlook of future research directions.

27.3 HISTORICAL BACKGROUND OF SKIN GRAFTING

Even though it is believed that skin grafting has been used by surgeons for more than 2500 years,[10] one of the first well-documented skin grafting experiments in a sheep model was published by Baronio[11] in 1804. The first report on the clinical application of skin grafting by using epidermal cells dates back to the end of the 18th century. Baronio's observations led Reverdin[12] to develop the so-called pinch-type grafts for human skin grafting. Mangoldt[13] described a technique in 1895 in which he scraped off the superficial layer of epithelium of the patients forearm with a scalpel until light wound secretion occurred. His concept was driven by the arguments that such a treatment concept would result in a decreased donor site morbidity and less scarring when compared with the Reverdin method, which was the standard at this time. He concluded, based on his extensive clinical experience and histological observations, that the cell clusters did stick better to the wound site than pieces of skin. It was attempted to improve the technique; however, the inconsistent clinical results prevented a broader application of this concept.

Almost at the same time as Mangoldt, Ljunggren[14] published a clinical report that harvested pieces of skin could be kept alive in a supplemented solution for a certain period of time in which it still had the potential to be used as skin graft. In the early nineteenth century, a number of researchers performed experiments with small skin pieces in form of explant cultures. These studies revealed that epithelial and connective tissue cells did proliferate out of the skin fragments when saline solution supplemented with different concentrations of glucose, serum, and ascetics were used. The development of standardized media that were supplemented with amino acids and peptides further improved the culture techniques. Subsequently, Pinkus[15] did perform a number of experiments which showed that basal precursor cells from skin fragments were responsible for the formation of a epidermal sheet and that differentiated keratinocytes become necrotic during that phase. Furthermore, those studies revealed that basal cell outgrowth was strongly influenced by the emergence of an intact dermis.

Nobel Prize Laureate Medawar was the first who successfully isolated proliferating epidermal cells.[16] He showed that keratinocytes isolated from dynamically cultured skin fragments could be implanted back to the donor without induction of a strong foreign-body reaction. Yet, further experiments and clinical trials revealed that the cell yield gained with the explant culture was not sufficient for large acute and chronic wound treatments. The studies executed by Billingham and Reynolds in 1952 showed that a small number of trypsinized keratinocytes did stay vital and could be further cultured.[17] However, his technique did not allow overcoming the main problem of gaining sufficient cell numbers to consider a clinical application. After a great number of trials with several substrates of synthetic and natural origin, Rheinwald and Green[18,19] succeeded to reproducibly culture a high number of nondifferentiated keratinocytes on a feeder layer of freshly irradiated mouse fibroblasts (3T3-J2 cells). This was the starting point for the clinical application of so-called cultured epidermal autografts (CEA). Despite several shortcomings of the CEA, which are discussed in detail below, the clinical society believed in the early 1970s that skin coverage in chronic and

acute wounds such as burns, chronic ulcers, scar revision surgery, and vitiligo could be effectively addressed by this technique.[20]

Barrandon and Green[21] did lay the groundwork on the characterization of CEAs from a molecular and cell biology point of view in 1984. They were the first to report that cells presented in a primary keratinocyte culture are also ranked in a differentiation hierarchy. Under clonogenic conditions, cells isolated from epidermal explants form different types of colonies, namely holoclones, meroclones, and paraclones. Only holoclones can be defined as epidermal stem cells, due to their exceptional self-replication ability of up to 180 times. The meroclone, which arises at the end of the differentiation cascade of holoclones, has a much shorter life span, and produces only colonies of meroclones and paraclones. The life span of paraclone is limited to a maximum of 15 cell divisions. Hence, the isolated and cultured holoclones progeny are mainly responsible for the production of an epidermal sheet.

Almost in parallel to the work of Rheinwald and Green, Yannas and Burke[22,23] as well as Bell[24,25] did start to work on dermal equivalents. The pioneers of matrix-based skin tissue engineering focused on the application of collagen as cell carrier. Collagen was a logical choice because it forms the most substantial group of structural proteins in connective tissue and represents about one-third of total body proteins. To date, more than 18 types of collagen have been identified, some of which have been completely characterized, and others only partially.[26]

Collagen type I is by far the most intensely studied polymer of natural origin. It has been used as a suture material for over a century. The mechanism, by which gut or other collagen implant materials degrade, is by sequential attack by specific enzymes. In most locations, the initial attack is by acid phosphatase with leucine amino peptidase activity increasing later during the degradation period. Collagenase is also thought to play a role in the enzymatic degradation of collagenous materials. In fact, the activity of collagenase is much higher for the processed, denatured protein than for the naturally occurring native collagen. The activity of collagenase can be reduced, however, if the collagen is crosslinked either with metal ions, which act as enzyme poisons, or with aldehydes. Consequently, treatment with glutaraldehyde, formaldehyde, or chromic salts greatly prolongs the degradation kinetics of these collagen-based materials.[27]

Histological as well as physical analysis of human skin samples led to the development of the so-called Integra Artificial Skin by Yannas and Burke,[22] the dermal matrix of which is made of reconstituted bovine tendon type I collagen/chondroitin-6-sulfate. The matrix design was based on the concept that cellular elements and supporting microvasculature of the host tissue are supposed to populate the collagen matrix and resemble normal connective tissue and a silastic layer maintains a nondegradable barrier function during the period of dermal regeneration.

The term *tissue engineering* was officially coined at a National Science Foundation workshop in 1988 to mean the application of principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue function (http://www.whitaker.org/95_annual_report/tissue95.html). Although cells have been cultured outside the body for many years, research has recently begun to develop complex three-dimensional tissue constructs that will ideally mature into fully functional tissues and organs. Today, clinical treatment concepts based on skin tissue engineering principles can be defined into six classifications (Table 27.1). A number of products (Table 27.2) based on especially the concepts 1 to 5 have been commercialized over the last two decades and will be discussed in the following section.

27.4 EPIDERMAL SKIN GRAFTS

27.4.1 Cultured Epidermal Autografts (CEA)

The cultivation and passaging of human keratinocytes with a sufficient yield from a clinical point of view was described by Rheinwald and Green[18] in the mid-1970s and thereafter was further

TABLE 27.1
Currently Used Matrix Technologies in Skin Tissue Engineering

1. Cultured Epidermal Autograft (CEA) which is consisting of a keratinocyte sheet grown on a irradiated fibroblast feeder layer
2. Keratinocyte (with or without irradiated fibroblast feeder layer) seeded on synthetic polymeric films, biodegradable and bioresorbable membranes made of natural and synthetic polymers
3. Keratinocytes are cultured in combination with a cell carrier, such as microspheres or beads which are made of fibrin glue, collagen, PLA/PGA etc.
4. A high number of cultured keratinocytes are mixed with fibrin glue directly before grafting and sprayed or suspended on the wound bed
5. Dermal graft — consisting of a three-dimensional scaffold (made of polymers of natural or synthetic origin) which is able to support cell attachment, proliferation, and ECM production is seeded and cultured with allogenic or autogenic fibroblasts
6. Skin Equivalent or True Skin graft — is made of matrices which are able to support both dermal and epidermal regeneration. Keratinocytes and fibroblasts are seeded and cultured directly or subsequently on 2-D (foils, membranes) and 3-D scaffolds (foams, textiles, cellular solids).

developed by others. This pioneering work of Green to successfully culture and subculture keratinocytes on a feeder layer of lethally irradiated mouse fibroblasts paved the way for clinical applications of cultured epidermal autografts (CEA). Subsequently, cultured keratinocyte grafting became established as a treatment option in severe burn injuries and in the clinical management of other wounds, such as chronic venous ulcers. Keratinocytes aimed to be transplanted were grown to confluency to form a dense epithelial sheet. The cultured epithelium was then detached as a coherent sheet from culture flasks by enzymatic treatment and transferred on a surgically prepared grafting bed to provide permanent coverage of excised third-degree burn wounds. The detachment of a coherent epidermal sheet is a difficult and time-consuming process. Enzymatic detachment of the epithelial sheet from plastic surfaces causes the sheet to shrink. Another problem is the inconsistency of graft take as well as the tendency to form blisters and scars due to exposure to shear forces. Hence, growing keratinocytes on a transplantable substrate that can be detached together with the epithelial sheet improves the CEA technique. One of the first improvements was attaching the CEA with surgical clips to a backing of petrolatum gauze. Using a suitable substrate that provides mechanical stability allows applications of epidermal grafts on areas subjected to mechanical stress and might improve the overall outcome of the regenerated tissue.

Acticel (Genzyme, Massachusetts), a nonautologous version of Epicel, was developed to have a product available "off the shelf." This product was never successfully commercialized in the U.S. However, allogenic epidermal grafts have been fruitfully studied by several European groups and have been commercialized recently as active wound dressing (CryoCeal, Xcellentis, Merelbeke, Belgium) for burns and ulcers. Today, an allogenic epidermal graft might be viewed as a biological active temporary wound dressing, due to the fact that allogenic cells only survive for a short period of time, during which epidermal keratinocytes may produce growth factors capable of accelerating the wound-healing process.[7]

The cultured epidermal autograft (CEA) has several shortcomings. It is only three to four cell layers thick and very fragile. The poor mechanical properties make it very difficult to handle a CEA from a clinical point of view, even if nondegradable matrix, such as a gauze, is used. It is detached as a coherent sheet from the culture flask by the application of dispase or thermolysin. This enzymatic process results in cell contraction and the graft shrinks to 30 to 50% of the cultured size. These difficulties were accompanied by the clinical observations that the CEA took poorly on deep chronic and acute wounds. In 2001 Horch et al.[28] edited a book in which five skin centers, which have been using the CEA as a routine treatment for at least 5 years, did report their most

TABLE 27.2
Clinically Available Tissue-Engineered Skin Grafts

Product	Company	Clinically Available Since	Type	Matrix Material	
				Epidermis	Dermis
Integra	Integra Life Sciences www.integra-ls.com	1984	Acellular dermal replacement	Polysiloxane membrane	Bovine tendon collagen, shark GAG
Alloderm®	Life Cell www.lifecell.com	2001	Acellular dermal replacement	—	Decellularized human cadaveric dermis
TransCyte®	Advanced Tissue Sciences www.advancedtissue.com	1997	Acellular dermal replacement	Silicone	Nylon mesh with nonviable foreskin fibroblasts
Dermagraft®	Advanced Tissue Sciences www.advancedtissue.com	1999	Cellular dermal replacement	—	Polyglactin mesh (Vicryl®) cryopreserved in combination with foreskin fibroblasts
Epicel®	Genzyme Corporation www.genzyme.com	1987	Cultured epidermal autograft	Viable autologous keratinocytes	—
Cultured human epidermis	Xcellentis nv www.innogenetics.com	2000	Cultured epidermal autograft	Viable autologous keratinocytes	—
CryoCeal	Xcellentis nv www.innogenetics.com	2000	Cultured epidermal allograft	Cryopreserved allogenic keratinocytes	—
Cultured epidermis	MeGA Tec GmbH www.mega-tec.de	2002	Cultured epidermal autograft	Viable autologous keratinocytes on bioresorbable membrane	—
BioSeed®-S	BioTissue Technologies AG www.biotissue-tec.com	2001	Autologous epidermal gel	Viable autologous keratinocytes suspension in fibrin glue	—
Apligraf	Organogenesis www.organogenesis.com	2001	Bilayered skin replacement	Viable allogenous foreskin keratinocytes	Bovine tendon collagen with viable foreskin fibroblasts
HYAFF™ Laserskin™	Fidia Advanced Bio-polymers, Via Ponte della Fabbrica 3/a, Abano, Terme (PD), I-35031	2000	Bilayered skin replacement	Viable keratinocytes on laser microperforated hyaluronan membrane (Laserskin™)	Viable fibroblasts on 3-D hyaluronan scaffolds (HYAFF™)
OrCel™	Ortec International www.ortecinternational.com	2001	Bilayered skin replacement	Bovine collagen	Bovine collagen

recent results. Overall, it can be summarized from these randomized clinical trials that the take rate of CEA is approximately 50 to 70%.

Based on the above, the key drivers to develop and apply different matrix material for the delivery of epidermal sheets were to improve not only the take rate but also wound healing by regaining skin functions such as sensitivity, elasticity, pigmentation, and most importantly accompanied by a low ratio of scarring. A number of research groups did concentrate in the 1990s on the development of new collagen matrices that can be used as an epidermal matrix.

27.4.2 Fibrin Glue

Over the last 10 years, fibrin glue has been extensively studied, experimentally as well as clinically as a delivery system for cultured keratinocytes as well as fibroblasts, and this work has been recently reviewed.[28,29] The first report of using fibrin glue in combination with noncultured keratinocytes was published by Hunyadi et al.[30] in 1988. In the 1990s fibrin glue has been widely used as cell carrier and matrix in skin tissue engineering.[31] Fibrin glue has been shown to improve the percentage of skin graft take, especially when associated with difficult grafting sites or sites associated with unavoidable movement. Fibrin, associated with fibronectin, has been shown to support keratinocyte and fibroblast growth both *in vitro* and *in vivo*, and may enhance cellular motility in the wound.[32] It also improved homeostasis and has a protective effect, resulting in reduced bacterial infection. Fibrin glue has also been shown to be a suitable delivery vehicle for exogenous growth factors that may in the future be used to accelerate wound healing.[33]

Skin tissue engineering concepts that use fibrin glue as matrix have been studied extensively by an interdisciplinary group at the Albert Ludwigs University Freiburg[34–36] and the Ecole Normale Superieur in Paris.[37,38] One of the clinical advantages is that fibrin glue suspension or the spraying method is easy to perform. Furthermore, allogenic fibrin glue is commercially available in most European countries or can be produced by using the patients own serum. Today, various clinical reports document the predictable wound healing if keratinocyte/fibrin glue suspensions (Figure 27.3) are applied for reepithelialization of regenerated deep partial- and full-thickness wounds.[26] The author's group has also shown clinically that the application of keratinocyte fibrin glue suspension as a spray facilitates the reepithelialization of a split-thickness burn wound on the external ear, with complex surface topography (Figure 27.4) (unpublished data).

Today, it is known that grafting of autologous melanocytes from normally pigmented donor skin can be used for repigmentation of achromatic macules in vitiligo.[39–41] A number of clinical centers did study grafts made of autologous melanocytes in fibrin glue on superficially laser dermabraded vitiligo lesions. The cellular suspension was grafted on vitiliginous lesions previously

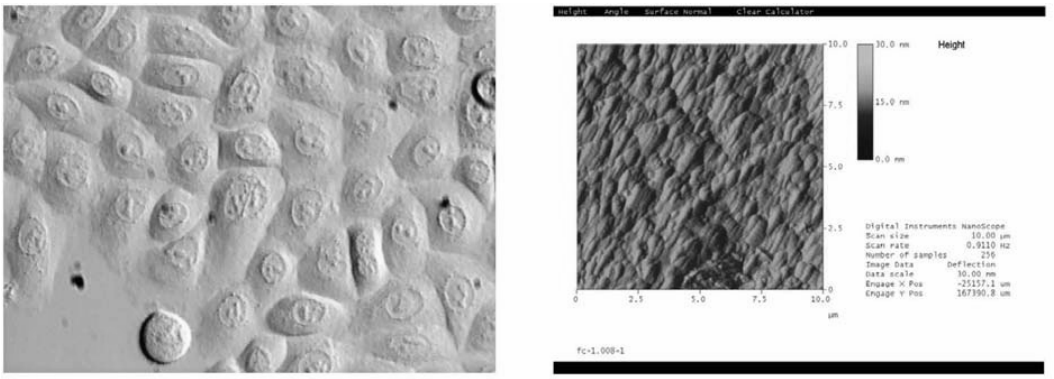

FIGURE 27.3 Light microscopy (left) and atomic force microscopy (right) images of human keratinocytes cultured on a fibrin glue-coated polycaprolactone membrane.

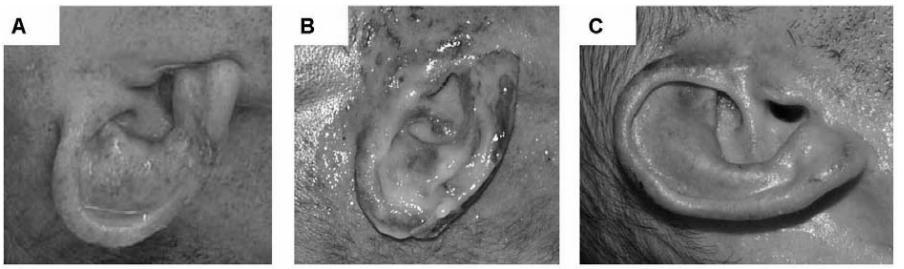

FIGURE 27.4 Split-thickness burn on external ear: (A) after debridement; (B) after spraying of keratinocyte–fibrin glue suspension; (C) at 3-week postsurgical follow-up.

dermabraded with a CO_2 laser. To improve take rate of the cellular suspension, human fibrin glue was used.[42]

Ronfard and Barrandon[35] reported most recently that single human keratinocytes invade the matrix and progress through it as rounded cells by dissolving the fibrin and thereby creating cylindrical and helical tunnels. A helical tunnel formation is strongly promoted by epidermal growth factor. The rate of migration of the cell through the path of a helical tunnel (up to 2.1 mm per day) is about sevenfold greater than through a cylindrical tunnel. The authors concluded that the ability of a keratinocyte to form a tunnel through a fibrin matrix permits a particular form of cell migration that may be adapted to the healing of a wound covered with fibrin. It has been shown by another group that keratinocytes move 20 times faster by tunnel formation into a fibrin matrix than when attached on native collagen I, the main matrix component of the dermis.[43]

27.4.3 Hyaluronan

In the late 1990s, an epidermal matrix made of a 100% hyaluronate acid derivative (Laserskin, Fidia Advanced Biopolymers, Italy) was commercialized. Hyaluronan (HA) or hyaluronic acid is a polysaccharide of the extracellular matrix (ECM). It is a main glycosaminoglycan (GAG) having many structural, physiological, and biological functions in the body. It is a linear and monotonous anionic polymer, which is heterogeneously distributed, in various soft tissues. Two modified sugars, glucuronic acid and *N*-acetyl glucosamine, form each of the disaccharide units. HA is a soluble molecule forming highly viscous solutions in water and interacts with binding proteins, proteoglycans, and growth factors, but also actively contributes to the regulation of the water balance acting on the osmotic pressure and low resistance and selectively sieving the diffusion of plasma and matrix proteins. In the joints, it behaves like a lubricant supporting the articular cartilage surfaces under shear stress. At a molecular level, HA acts as a scavenger molecule for free radicals. In the last decade, the use of medical grade HA has been applied widely in orthopedic surgery, corneal transplantation, and treatment of cataract, intraocular lens implantation, treatment of vitro-retinal diseases. In addition, it was shown that HA can improve wound healing due to degradation products which induce endothelial cell proliferation and angiogenesis.

HA-based scaffolds have been studied by a number of tissue engineers due to its excellent cell and tissue compatibility.[44,45] However, water solubility, rapid resorption, and short residence time at the site of implantation did lead biomaterial scientists to modify its molecular structure to fabricate a scaffold material with sufficient physical properties for skin tissue engineering. Crosslinking and coupling reactions were two of the ways considered for obtaining a material with better mechanical properties. These chemical modifications were applied either to trap HA chains within a net of crosslinked proteins, or to create covalent bonds between HA chains. The production of all these derivatives was driven by a concept similar to that which led to the production of crosslinked collagen. However, in a number of cases, concern has been expressed for the potential toxicity of some of the crosslinking agents utilized, such as glutaraldehyde, formaldehyde, and isocyanates.

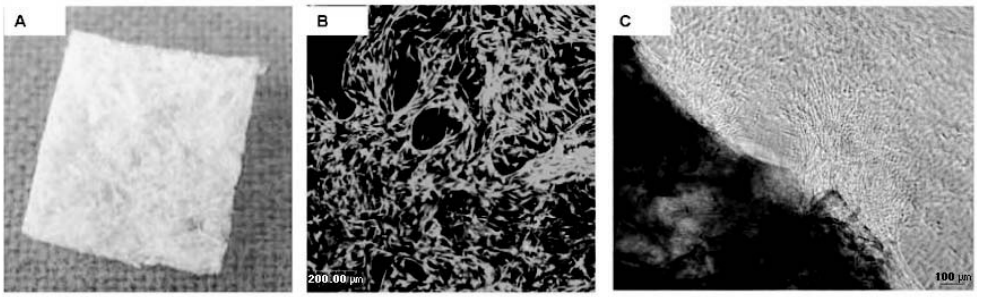

FIGURE 27.5 A collagen/hyaluronan membrane was evaluated as a matrix for human dermal fibroblast culture. (A) Macroscopic view of collagen/hyaluronan membrane, (B) confocal laser microscopy, and (C) light microscopy showed attachment and proliferation of cells in the membrane, over 4 weeks of culture.

Therefore, a new type of HA was obtained by creating crosslinking bonds by directly esterifying a certain percentage of the carboxyl groups of glucuronic acid along the polymeric chain with hydroxyl groups of the same or different hyaluronan molecules. Once esterification of the polymer has been obtained, the material can easily be processed to fabricate different scaffold types such as membranes, sponges, and microspheres via extrusion, lyophilization, or spray drying. Once wet, the benzylester loses part of its mechanical strength like many other natural polymers. However, under *in vitro* cell culture conditions, the material maintains its structural integrity for up to 3 weeks and does not shrink as collagen-based materials do.[46,47]

The work on the various formulations of hyaluronan-based matrices, in respect to skin tissue engineering, have been included in a number of reviews.[48,49] It has been reported that an advantage of HYAFF™ is the good cell attachment and proliferation of fibroblasts and chondrocytes, even in the absence of any protein coating or surface treatment often required by those other matrices made of synthetic polymers. The gained knowledge about processing of large sheets of HYAFF did lead to the development of a matrix for epidermal sheets (Laserskin, Fidia Advanced Biopolymers, Italy). The membrane has microperforations (40 microns, 6.000 perforations/cm^2), which allow the keratinocytes to communicate and grow toward the host tissue. Holes of 0.5 mm function as drainage for the wound exudates.[50,51] However, keratinocytes do not sufficiently attach and proliferate on the Laserskin. Therefore, the manufacturer recommends culturing the keratinocytes with a fibroblast feeder layer. Harris et al.[52] has demonstrated the efficacy of a tissue-engineered membrane/cell construct by using Laserskin for clinical transplantation of autologous keratinocytes.

Bakos et al.[53] did develop a collagen/hyaluronan membrane, to combine the material properties of a protein and polysaccharide. According to their results, interactions of these two polymers are very strong and result in improved degradation kinetics as well as mechanical properties. The properties can be influenced from the material science point of view by chemical crosslinking using glyoxal and starch dialdehyde. The composites of collagen–hyaluronic acid have been used successfully in a number of clinical studies as epidermal matrix. The authors' group has evaluated the collagen/hyaluronan membrane as a matrix for supporting the development of a dermal-like tissue *in vitro*. The matrix was cultured and encapsulated in a human dermal fibroblast sheet over 4 weeks of *in vitro* culture. Confocal laser microscopy and histology showed that the material supported the attachment and proliferation of human dermal fibroblasts to form a dermal-like neotissue (Figure 27.5). Contraction of the material over the culture period was minimal.

27.4.4 Chitosan

Chitosan is a semicrystalline polymer and the degree of crystallinity is a function of the degree of deacetylation. Crystallinity is maximum for both chitin (i.e., 0% deacetylated) and fully deacetylated (i.e., 100%) chitosan. Commercially available medical grade materials have degrees of deacetylation

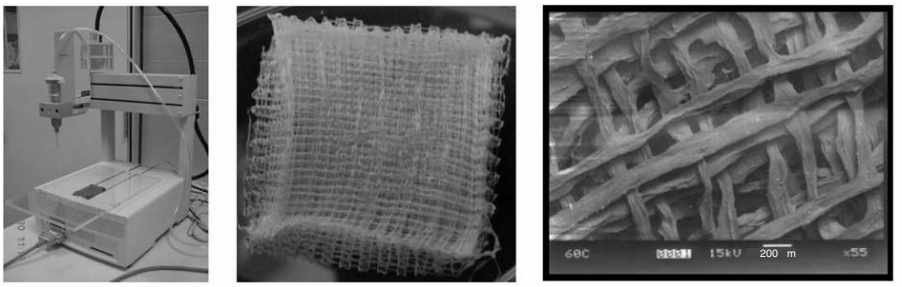

FIGURE 27.6 The RPBOD system for manufacturing 3-D chitosan scaffolds. (A) A three-axis robotic arm; (B) top view of scaffold made by dispensing dissolved chitosan, layer by layer, using the in-house designed and fabricated RP system; (C) scanning electron micrograph of chitosan scaffold, showing regular pore architecture with high interconnectivity.

ranging from 50 to 90%. Structurally, chitosan is a linear polysaccharide consisting of[14] linked *D*-glucosamine residues with a variable number of randomly located *N*-acetyl-glucosamine groups. It thus shares some characteristics with various GAGs and hyaluronic acid present in articular cartilage. Since GAG properties include many specific interactions with growth factors, receptors, and adhesion proteins, this suggests that the analogous structure in chitosan may also have related bioactivities. In fact, chitosan oligosaccharides have been shown to have a stimulatory effect on macrophages during wound healing. Formation of normal granulation tissue, often with accelerated angiogenesis, appears to be the typical course of healing when chitosan is used as implant. *In vivo*, chitosan is degraded by enzymatic hydrolysis.

Because of the stable, crystalline structure, chitosan is normally insoluble in aqueous solutions above pH 7. However, in dilute acids, the free amino groups are protonated and the molecule becomes fully soluble below ~ pH 5. The pH-dependent solubility of chitosan provides a convenient mechanism for processing. Hence, one of chitosan's most promising features is its excellent ability to be processed into porous structures for use in cell transplantation and tissue regeneration. Freezing and lyophilizing chitosan acetic acid solutions can form porous chitosan structures.

The author's group do design and fabricate chitosan scaffolds (see also Section 27.6 of this chapter) via a solid free-form fabrication technology (Figure 27.6). To overcome current limitations for treating mustard-burn-induced septic wound injuries, a nonadherent matrix with sustained antimicrobial capability has been developed by Loke et al.[54] and Yan et al.[55] The wound dressing consists of two layers; the upper layer is a carboxymethyl-chitin hydrogel material, while the lower layer is an antimicrobial impregnated biomaterial. The hydrogel layer acts as a mechanical and microbial barrier, and is capable of absorbing wound exudates. Furthermore, the group developed films based on chitosan-alginate coacervates. The films exhibited good attachment and proliferation of human fibroblasts, suggesting that they can be further explored as matrix for skin tissue engineering. A number of researchers have studied chitosan-based scaffolds in various tissue engineering applications. The application of chitosan as matrix material in tissue engineering applications has been reviewed in detail elsewhere.[56]

27.4.5 Synthetic Polymers

From a more general material point of view, aliphatic polyesters have physical and chemical properties which make them a potential candidate as matrix materials for tissue engineering applications.[57] The advantages of synthetic bioresorbable polymers over natural ones include better mechanical properties, more readily processible into a variety of micro- and macroarchitectures as well as shapes using standard polymer processing techniques, and more easily modified degradation

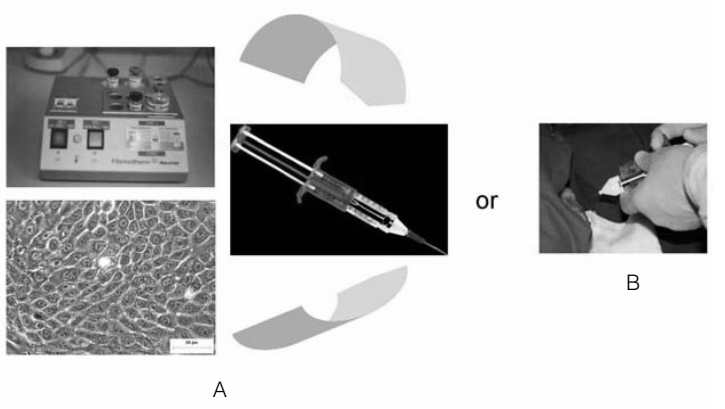

FIGURE 27.7 (A) Perforated polycaprolactone film with hole size of ca. 100 μm. (B) Fibrin glue (Tisseel kit, Baxter) is used with an injection syringe or as a spray to coat films.

and resorption profiles. However, due to the superior cell compatibility and tissue-integration function in benchmarking against ordinary synthetic polymers, in the past, collagen, polysaccharides, and other polymers of natural origin has been the material of choice as matrices for epidermal skin tissue engineering.

No successful epidermal skin grafts using bioresorbable polymers of synthetic origin as the matrix material have been reported to date, although, the potential of polycaprolactone (PCL) has been highlighted as far back as 1974.[58] Sutures and drug delivery devices made of PCL and its copolymers have FDA approval for many years and a number of new sutures and medical devices have been approved in Japan and Europe.[59]

The key to an ideal epidermal matrix could depend on the ability to fabricate a synthetic polymer membrane as thin as possible while having suitable mechanical properties and a good cell and tissue compatibility. Such an ultra-thin matrix has a number of significant advantages. First, a thin graft would ensure a high degree of flexibility so as to conform easily to the morphology of the anatomy of the wound site. This would help to maximize contact between the graft and the underlying tissue, thereby improving graft acceptance. Second, a thin graft would allow for maximum biochemical interaction between the graft and the underlying tissue. Diffusion of nutrients, waste products, and growth factors would be more efficiently transported. Third, an ultra-thin polymer matrix would limit the mass of the polymer matrix implanted and hence minimize the quantity of degradation by-products so that the foreign-body reactions are minimal.

The interdisciplinary group[60] of one of the authors of this review has developed a biaxially stretched, PCL film, which can be fabricated in a thickness of 5–15 μm. The ultra-thin film has a novel surface microtexture combined with a high tensile strength. The results of a number of *in vitro* and *in vivo* studies indicate that biaxially stretched PCL films support the attachment and proliferation of human epidermal keratinocytes as well as fibroblast-like cells.[61–63] Perforated and fibrin glue coated membranes have been developed recently (Figure 27.7).

27.5 DERMAL SKIN GRAFT

In recent years, it has become increasingly apparent that many of the problems encountered upon application of CEA — e.g., scar contractions or unstable attachment of the CEA to the underlying tissue — could be attributed to the absence of a dermal substrate. The prognosis of patients with severe burn trauma is more favorable the earlier the necrectomy and definitive wound coverage can be performed.[64] Hence, the most urgent aim in the treatment of burns is the quick reconstruction of a closed epidermis, as this eliminates the immediate vital threat of losing energy, protein,

electrolytes, and fluid. In addition, the epidermis is the most effective barrier against infection and thus against the danger of sepsis, which is still the most frequent cause of death after burns. Already in the early stages of treatment, however, it should be considered that an epidermal coverage alone is by no means sufficient to prevent functional and aesthetic impairment of the new skin through lack of mechanical stability or scar contractions. Hence, the application of a wound dressing which aims at restoration of the dermal tissue is a condition sine qua non.

Biobrane (Dow B. Hickam, Inc, Sugarland, TX) is a bilaminate material comprising a nylon film coated with a layer of silicone rubber. The silicone is coated with porcine peptides derived from type I collagen. The presence of the porcine collagen is reported to promote fibrovascular ingrowth, recruiting fibroblasts and endothelium into the wound site. The product has been used unseeded for the treatment of donor sites, noninfected superficial partial thickness burns, and as a substitute to cadaver skin prior to burn grafting.[65] Although the product was originally developed as a wound dressing, researchers and clinicians did explore the potential of Biobrane as a cell carrier. Experimental results reported thus far use neonatal fibroblasts, grown on Biobrane, and grafted onto athymic mice. When comparing seeded and nonseeded Biobrane, the authors concluded that "the seeded variant provided better adherence to the wound bed, a reduction in inflammation, and increase in vascularization." TransCyte (Advanced Tissue Sciences, La Jolla, CA) is composed of human newborn fibroblasts which are cultured on the nylon mesh of Biobrane; the thin silicone membrane bonded to the mesh provides a moisture vapor barrier for the wound. This matrix has been used for temporary coverage of partial-thickness or full-thickness burns.[66]

Integra Artificial Skin was originally developed by Yannas and Burke. The lower layer of Integra is made of a matrix of crosslinked bovine collagen and shark-derived chondroitin-6-sulfate. It has a pore size of between 70 and 200 microns and regular matrix architecture similar to that of the dermal layer in skin. A temporary polysiloxane layer maintains a permanent barrier function normally provided by a cornified skin epidermis during the period of wound healing. In clinical practice, once the wound site is stable, the silastic layer is peeled away to leave a vascularized neo-dermis suitable for epidermal grafting by using widely meshed autologous split-thickness skin graft. In one of the first published multicenter studies involving 106 patients, Integra had a median take rate of over 80%.[67] It was noted, however, that in any one patient the artificial skin would either achieve a high take rate or a very low take rate. It was also reported that the take rate of individual centers improved as surgeons became more experienced at using the material. There is, however, no consensus on the best protocol for the use of this material and, as a consequence, several different techniques are in clinical use.[68] Originally developed as a wound dressing, recently, studies have been executed in which fibroblasts and epidermal cells have been seeded into the collagen matrix of Integra.[69,70]

Dermagraft (Advanced Tissue Sciences, La Jolla, CA) consists of a synthetic, knitted PLA/PGA Vicryl mesh (Johnson & Johnson, NJ) that is seeded and cultured with foreskin fibroblasts (passaged up to eight times). The skin graft is cryopreserved after culturing the scaffold/cell construct for up to 20 days in a closed bioreactor system. In most of the earlier studies, Dermagraft was used for the treatment of foot ulcers in diabetic patients. Today, a number of clinical trials investigate the performance of this type of tissue-engineered skin graft in partial-thickness or full-thickness burns.[71,72]

Over the last decade, several attempts were made to develop biotechnological methods of processing human dermis to gently remove all cells while preserving undamaged the extracellular matrix. AlloDerm (LifeCell Corp., The Woodlands, TX) is derived from human skin which is collected and sliced into thin sheets. A super dry lyophilization method allows removal of the epidermis and all dermal cells without major disruption of the collagen matrix. It is packaged as a freeze-dried sheet and is easily rehydrated at the time of surgery. Its lead indication is for the treatment of foot ulcers in diabetic patients, but it is also evaluated in clinical trials for the treatment of partial-thickness or full-thickness burns. XenoDerm (LifeCell Corp., The Woodlands, TX) is

fabricated the same way as Alloderm, only from pig dermis. It is currently under clinical investigation, too.

Autologous fibroblasts were cultured in fleece scaffolds made from benzyl esters of hyaluronic acid and applied clinically onto cutaneous lesions. The authors concluded that their preliminary results suggest that autologous fibroblast culture in hyaluronan-derived scaffolds may be successfully grafted in diverse cutaneous pathologies and constitute a suitable bed for further epidermal implantation.[73] In a study by von Heimburg et al.,[74] sponges and nonwoven carriers based on HYAFF were compared with collagen sponges. The authors conclude that HYAFF sponges supported the expansion and differentiation of the adipose precursor cells. This carrier is superior to the nonwoven carrier with regard to adiposity differentiation and superior to the collagen sponge with regard to cellularity.

27.6 SKIN EQUIVALENT

The successful cultivation of human keratinocytes first was described by Rheinwald and Green in the mid-1970s and was thereafter defined by many other clinical and research groups. After this pioneering work, cultured keratinocyte grafting became established as a treatment option in severe burn injuries and in the clinical management of other wounds, such as chronic venous ulcers. Even though keratinocyte culture techniques have improved with the use of potent stimulators of their proliferation and colony-forming capacities, resulting in the development of confluent keratinocyte laminae up to a thickness of 10 to 15 cells, their clinical use is often unsatisfactory.

Therefore, the presence of a scaffold supporting the keratinocyte layers might overcome several of the problems, such as fragility, handling, contraction, and, with an appropriate preparation of the wound bed, improve the take rate, otherwise unpredictable before grafting. However, several studies have shown that the presence of a dermal layer is of great importance in the regulation of the growth and differentiation of cultured keratinocytes, too. Today, it is widely accepted in the skin tissue engineering community that fibroblasts embedded in their extracellular matrix (ECM) constitute a permissive and regulatory three-dimensional environment in which keratinocytes can express also *in vitro* the necessary adhesion molecules and ECM proteins.[75] Conclusively, it might be possible with a true skin equivalent to clinically regenerate a dermal-epithelial junction and, in particular, a basement membrane, which improves the cultured skin strength and resistance. In addition, the aim of performing a single-stage grafting procedure is highly beneficial in terms of replacing the requirement for traditional split-skin grafts.

Apligraf (Table 27.2) is the first FDA-approved composite skin graft which was originally designed by Bell et al.[24] It consists of a type I collagen gel and allogenic fibroblasts mixture on which a confluent layer of allogenic keratinocytes is cultured. This product has been studied extensively over the last 10 years.[76,77] Long et al.[78] applied Apligraf on 30 chronic lower extremity ulcers and found that 80% of these wounds healed completely, with an average healing time recorded at 6 months. It is stated that the cell lines obtained from foreskin are nonimmunogenic and therefore present little potential for rejection. However, it seems to be that there is, today, consensus in the scientific community that long-term survival of allogenic keratinocytes and fibroblasts in deep dermal wounds is limited. Tissue-engineered allografts may, however, have some benefit, as viable allogenic cells may deliver biologic mediators (growth factors such as TGF-beta and IL-1) capable of accelerating the repair process.

Another attempt to engineer a living skin equivalent, a so-called composite cultured skin graft (OrCel, Ortec International, Inc., TX), consists of allogenic fibroblasts and keratinocytes seeded on opposite sides of bilayered matrix of bovine collagen. There are limited clinical data available for this product,[79] but large clinical trials are ongoing. Still et al.[80] demonstrated that Orcel accelerated healing time and reduced scarring, compared to a standard dressing (Biobrane-L), when grafted onto split-thickness wounds.

Collombel and his group[81–83] have published several papers highlighting the clinical potential of a matrix which is fabricated out of three different natural polymers. The foam-like matrices are obtained by lyophilization of a solvent-based mixture of collagen, chitosan, and chondroitin-6-sulfate. Such a composite material reveals improvement of mechanical strength when compared to the original Yannas and Burke matrix due to the introduction of ionic bounds between the collagen and chitosan. Foams of a thickness of up to 5 mm have been studied *in vitro* and *in vivo* by seeding keratinocytes on top of the precultured foam/fibroblasts construct. Building on the work of Collombel et al., Braye et al.[84] cocultured fibroblasts and keratinocytes on a substrate composed of collagen–glycosaminoglycan–chitosan to treat a large skin defect in immunosuppressed female pigs. Full-thickness skin resections of 50 to 100 cm^2 were treated with a tissue-engineered graft on the dorsa of the animals with good wound regeneration.

The aim of a study by Zacchi et al.[85] was to develop and characterize a skin equivalent composed of HA matrices. Keratinocytes were seeded and cultured for 15 days on a hyaluronic acid-derived membrane and fibroblasts in a foam-like matrix made of the same material. Then, the membrane was placed on the foam and cocultured for an additional period of 15 days. Results showed that human fibroblasts and keratinocytes can be cultured on hyaluronic acid-derived scaffolds and that the protein expression is similar to that found in normal skin. The data from this and other studies[86] suggest that this skin equivalent might be useful in the treatment of both burns and chronic wounds.

Another natural polymer that has been commonly studied as a matrix material for skin regeneration[87] but exists outside the human body in nature is chitosan (see previous discussion). Chitosan can be easily fabricated into various porous structures, including membranes, blocks, tubes, and beads.[88,89] The drawbacks of conventional scaffold fabrication techniques have encouraged a number of research groups to utilize rapid prototyping. This method, also often referenced to solid free-form fabrication, offers the potential to design and fabricate scaffolds with novel properties and characteristics.[90] The author's group uses a fabrication process that resembles a 3-D plotting technology[91] to manufacture dermal substrates. The fabrication technique offered by the RPBOD system allows chitosan to be used in a variety of compositions and concentrations while a custom-made developed software (made up of a slicing and dispensing program) complements the system by allowing users to generate geometrical data of 3-D scaffolds through user-friendly interfaces. The aim of the skin tissue engineering group at the National University of Singapore is to use a chitosan scaffold in combination with a perforated PCL film for culturing a true skin equivalent (Figure 27.6).

27.7 FUTURE PERSPECTIVES AND RESEARCH DIRECTIONS

Results from the use of polymers of natural origin, such as cell matrices for skin tissue engineering, have been encouraging. Notable drawbacks, including cost, storage, preservation, immunologic reactions, and, particularly, potential infections, associated with the use of natural polymers of allogenic or xenogenic origin have promoted the development of scaffolds made of new types of synthetic polymers. A block copolymer of poly(ethylene-glycol terephthalate) and poly(butylene terephthalate) has been shown to support the proliferation of human dermal fibroblasts and keratinocytes.[92] Foam-like matrices made of poly-*L*-lactic acid (PLA) did support the proliferation of human dermal fibroblasts[93] too, and several other synthetic polymer textile meshes are currently being investigated. LaFrance and Armstrong[94] cultured dermal fibroblasts and keratinocytes on the surface of PLGA microspheres. Using the porcine model, they injected microspheres cultured with fibroblasts and epithelial cells into full-thickness wounds and found that cell-coated spheres promoted more rapid and more natural wound healing than uncoated microspheres. One of the concerns with all skin grafts involves the method and ease of surgical application. Microspheres provide several advantages since they allow easy handling, do not need to be sutured to the wound site, and do not have to conform to the wound bed. One major disadvantage of this material, however,

is that the microspheres lack the ability to control fluid loss from the wound surface and to provide a barrier to infection.

In conclusion, one of the major disadvantages of using synthetic bioresorbable polymers as matrices in skin tissue engineering is their poor surface properties in respect to promote attachment, proliferation, and differentiation of epidermal cells. The application of microfabrication techniques, which allow the design and fabrication of so-called biomimetic surfaces, offers interesting tools to overcome the current drawbacks.

27.7.1 Skin Precursor Cells

In the past, stem cells in the adult have traditionally been thought to be restricted in their differentiated and regenerative potential to the tissues in which they reside. However, recent findings suggest that stem cell biology may be more complex than originally anticipated.[95–97] The increasing number of papers reporting about formerly unknown function, structure, and plasticity of different type of stem cells has spawned a major switch in the perception of their nature and their potential application in tissue engineering concepts. The ability to isolate a subset of stem cells from the skin or adipose tissue, which inherit the most extensive replication and differentiation potential, could naturally be of utmost importance for new skin tissue engineering strategies.

It was known for almost two decades that keratinocytes follow a specific differentiation path as they transit through the suprabasal layers toward the skin surface. More recently, a number of groups[98–102] reported that the upper region of the outer root sheath of vibrissal follicles contains multipotent stem cells that respond to morphogenetic signals to generate multiple hair follicles, sebaceous glands, and epidermis, i.e., all the lineages of the hairy skin. At the time when hair production ceases and when the lower region of the follicle undergoes major structural changes, the lower region contains a significant number of clonogenic keratinocytes, and can then respond to morphogenetic signals. This demonstrates that multipotent stem cells migrate to the root of the follicle. Moreover, this result indicates that the clonogenic keratinocytes are closely related, if not identical, to this type of skin stem cells. Fu et al.[103] reported in a clinical study that their histological analysis showed that skin stem cells are capable to revert *in vivo* from differentiated to undifferentiated stem cells. Hoeller et al.[104] showed the potential of freshly plucked hair follicles as cell source when tissue engineering a true skin equivalent.

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28 Skin Tissue Engineering Part II — The *In Vitro* Evaluation of Natural and Synthetic 3-D Matrices as Dermal Substrates

K. W. Ng, H. L. Khor, and Dietmar W. Hutmacher

CONTENTS

28.1 INTRODUCTION

Tissue engineering skin, based on the concept of a cell–matrix construct, represents the most successful clinical application in regenerative medicine today.[1,2] Skin replacement and regeneration products were commercially available since the 1990s. These products include epidermal replacements such as the Laserskin®,[3] dermal replacements such as the Dermagraft®,[4] and also bilayered skin replacements such as the Apligraf®[5] and Orcel®.[6] Dermagraft (Advanced Tissue Sciences Inc., La Jolla, CA), a dermal replacement product, uses a synthetic polyglactin mesh as a substrate for

neonatal fibroblasts. The use of Dermagraft in clinical treatment of diabetic foot ulcers has yielded a 50% rate of complete healing compared to 8% in controls.[7] However, repeated applications are needed to increase the success rate of using Dermagraft, and a split thickness graft needs to be applied to complete the healing. Apligraf (Organogenesis Inc., Canton, MA), a commercially available bilayered skin replacement has a viable, fibroblast-containing bovine collagen type I matrix as the dermal component, onto which keratinocytes are seeded to form an epidermal sheet.[8] This skin equivalent was shown to be morphologically, biochemically, and metabolically similar to human skin.[9] Clinically, Apligraf has been used successfully in the treatment of chronic diabetic foot ulcers, with improved frequency of closure (75% vs. 41% in controls) and reduced time-to-wound closure shown (median time 38.5 days vs. 91 days in controls).[10] However, Apligraf has a flat dermoepidermal junction as opposed to the undulating ridges found in native skin, which could have the effect of lowering its shear strength and stiffness. In addition, Apligraf has a relatively short shelf life of 5 days and the use of an animal-derived scaffold could pose a risk of inter-species pathogen transfer. Another commercialized composite living skin equivalent is Orcel. Similarly to Apligraf, Orcel[6] is composed of a collagen sponge seeded with allogeneic epidermal and dermal cells. The company (http://www.ortecinternational.com) claims that the cells secrete growth factors and cytokines normally found in acute human wounds and are believed to have a beneficial role in promoting tissue repair. There is limited clinical data available for this product, but extensive clinical trials are ongoing.

A number of issues, which contribute to the limitations of the above discussed cell matrix constructs, still need to be addressed. Limitations of these products to be applied in large clinical scale include poor mechanical properties, short shelf life, low engraftment efficiency, high cost, the lack of native skin functions and characteristics such as sensation, presence of hair follicles and sweat glands, and the uncertainty of long-term host immunological response where xenogenic and allogenic matrices as well as allogenic cells had been used.

In choosing a matrix material for the purpose of dermal regeneration, attention must be drawn to a best possible combination of biological, chemical, physical, and mechanical characteristics of the matrix to ensure success. Not only must the matrix be able to induce the appropriate biological responses in terms of fibroblast attachment, proliferation, and ECM production, it must also be able to provide the right physical environment to induce the formation of a viable neo-dermis, in terms of cell and ECM distribution as well as size, shape, and morphology of the dermis required. In addition, it must serve the purpose of being a temporary scaffold, providing the necessary mechanical stability to withstand the contraction forces exerted by myofibroblasts *in vitro* and *in vivo*, while the neo-dermis matures. Based on our literature review,[2] we came to the conclusion that no single material has proven to be superior for the purpose of dermal regeneration.

The underlying goal of our skin tissue engineering research program is to develop a bilayered full-thickness skin equivalent which also contains blood vessels, nerves, and sweat glands (Figure 28.1). A bioresorbable film fabricated using poly(ε-caprolactone) (PCL) has been developed which has the potential to be used as an epidermal carrier.[11] This film has the advantages of being ultrathin (5–15 μm) and highly flexible and having good mechanical properties (tensile strength 55 MPa). The biocompatibility and capability in supporting cell attachment and proliferation of such a PCL film and a with fibrin glue coated PCL membrane has been demonstrated in cell culture studies using human dermal fibroblasts[12] and human keratinocytes.[13] The aim of this study was to compare the suitability and efficacy of natural and synthetic matrices as dermal substrates. The study forms part of a broader series of assessment of different biomaterials as dermal substrates.

28.2 MATERIALS AND METHODS

28.2.1 Substrate Preparation

Two commercially available collagen-based matrices were used in the study. Acellular dermal graft, Alloderm®, from LifeCell Corporation, New Jersey, and equine collagen foam, TissuFleece®, from

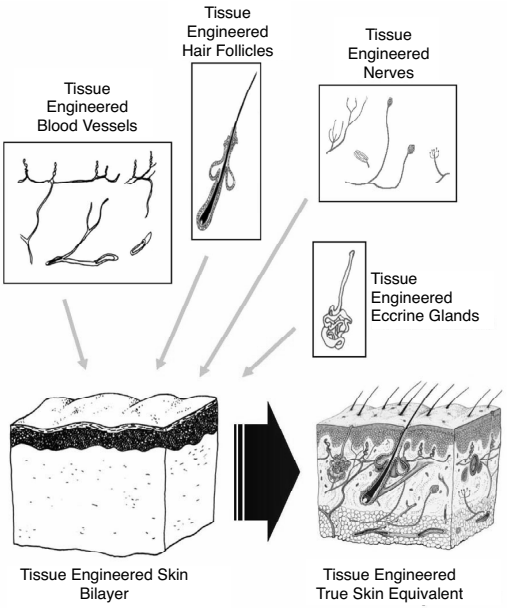

FIGURE 28.1 Schematic concept of developing a true bilayered skin equivalent.

Baxter Healthcare Corporation, Illinois. High purity and high viscosity chitosan with a deacetylation of more than 80% was obtained from CarboMer, Inc., Washington, D.C., while poly(lactic-co-glycolic acid) (PLGA-10/90) fiber was supplied by Shanghai Tianchun Biomaterials Company Ltd., Shanghai, China. This PLGA-10/90 fiber is similar in composition to commercially available Vicryl® sutures and meshes. Poly(ε-caprolactone) (PCL) fibers were drawn in-house from PCL pellets (catalog no. 44,074-4) purchased from Aldrich Chemical Company Inc., Wisconsin.

Figure 28.2 shows the gross morphology of the specimen groups prior to cell culture. Alloderm was cut to size and used as recommended by the supplier. TissuFleece was cut to size and hydrated prior to use. The PLGA-PCL mesh was knitted from continuous fiber yarns of PLGA and PCL, as previously described.[14] Briefly, 60 continuous PLGA single fibers of 20 μm diameter were combined with 4 continuous PCL single fibers of 80 μm diameter to form a PLGA-PCL yarn, which was knitted into a mesh using a Silver Reed SK270 Knitting Machine (Suzhou Zhenzuo Mechanical Instrument Company Ltd., China). The meshes were folded and fused by heat along the edges to form three-layered meshes of approximately 3 mm thick and a porosity of approximately 97%. The three-layered meshes were subsequently cut to size and sterilized in 70% ethanol (J.T. Baker, New Jersey) for 2 hours prior to usage. The meshes were flexible and highly porous, with loosely bundled fibers not more than 100 μm apart. Inter-yarn spaces were relatively large, ranging from 500–1000 μm wide. Chitosan scaffolds were fabricated via a three-axis robotic arm dispensing system developed in-house.[15] Briefly, a viscous gel of chitosan in acetic acid was dispensed using a pneumatic dispenser at a pressure of 1.5 bar. Coordinated movement in the *x*-, *y*-, and *z*-axes allowed the gel to be extruded layer by layer into a petri dish containing sodium hydroxide (NaOH) solution, which crosslinked the chitosan to produce solid, regular 3-D scaffolds. NaOH was removed by rinsing the scaffolds with deionized water. The scaffolds were then freeze-dried. Prior to use, they were hydrated, cut to size, and sterilized in 70% ethanol. The scaffolds were 3 mm thick, with porosity higher than 90% and fully interconnected macro pore architecture.

28.2.2 Cell Isolation, Culture, and Seeding

Human dermal fibroblasts (HDFs) were derived from enzymatic digestion of human skin samples with 2 mg/ml collagenase type I (Roche, Switzerland), overnight at 37°C. Isolated cells were

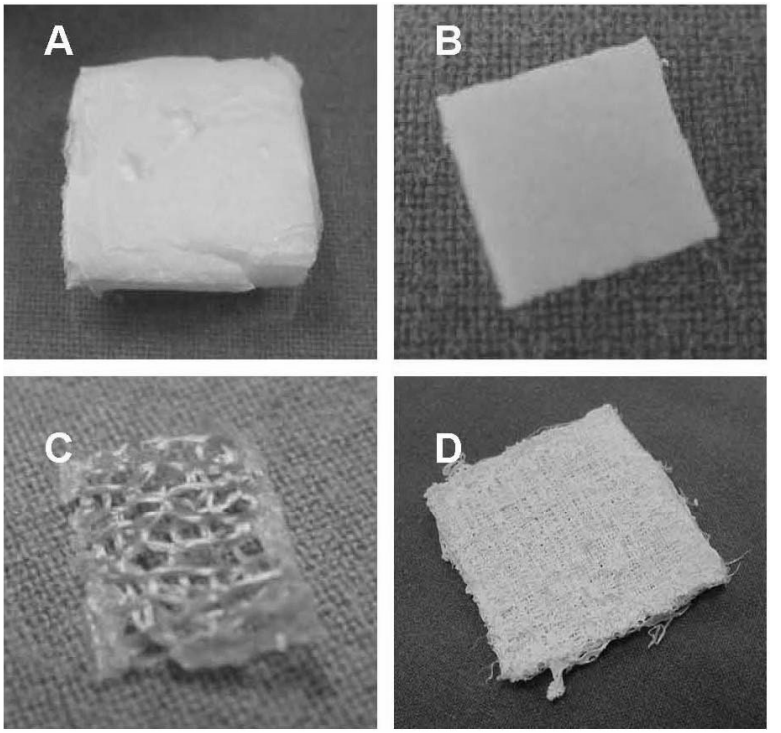

FIGURE 28.2 Gross morphology of four specimen groups: (A) TissuFleece, (B) Alloderm, (C) PLGA-PCL mesh, (D) chitosan scaffold.

subsequently cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, New York) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone, Utah) and 1% Penicillin-Streptomycin solution (Sigma, St. Louis, MO, USA) in culture flasks. For routine culture and experiments, cells were placed in a self-sterilizable incubator (WTB Binder, Tuttlingen, Germany) at 37°C in 5% CO_2, 95% air, and 99% relative humidity, with medium changed every 2 days. HDFs at their third passage were used in the experiment. Cells were trypsinized, resuspended in complete DMEM, counted, and pipetted onto the specimens at a density of 500,000 cells per cm^2.[16] The specimens were transferred to the incubator in 24-well plates and left for 90 min to allow for cell attachment. Each well was then carefully filled along the walls of the individual wells with 1.5 ml complete DMEM with 25 µg/ml *L*-ascorbic acid (Sigma, St. Louis, MO) to submerge the specimens. Characterization of the specimens was carried out at 1, 2, and 3 weeks postseeding.

28.2.3 Cell Morphology and Viability

Cell morphology was analyzed with phase contrast light microscopy (PCLM) and electron microscopy (EM) while cell viability was determined with confocal laser microscopy (CLM). For scanning electron microscopy (SEM), specimens were fixed in 2.5% glutaraldehyde (Merck, Germany) for 4 h at 4°C. Week 1 specimens were dehydrated in a graded ethanol series, dried, gold-coated with a Jeol JFC-1200 fine coater, and viewed under a Philips XL30 FEG scanning electron microscope. Week 2 and 3 specimens were viewed under a Philips XL30 TMP environmental SEM (ESEM), at a pressure of 3 Torr, directly after fixation. To assess cell viability, specimens were first incubated at 37°C with 2 µg/ml fluorescein diacetate (FDA, Molecular Probes Inc., Oregon) in phosphate-buffered saline (PBS) for 15 minutes to stain viable cells green, and then placed in 0.1 mg/ml propidium iodide solution (PI, Molecular Probes Inc., Oregon) for 2 min at room temperature to

stain nonviable cells' nuclei red. The samples were viewed under a Confocal Laser Microscope (Olympus IX70-HLSH100 Fluoview).

28.2.4 Cell Proliferation

Cellular proliferation characteristics on both the 3-D specimens and the 2-D cultures on the bottom of the well plates was determined via [^{3}H]-thymidine labeling and MTS metabolic assay (n = 5). The specific activity range of [^{3}H]-thymidine used was greater than 10 Ci/mmol (NEN Life Sciences, Massachusetts) and a concentration of 3.3 μCi/ml in complete culture medium was introduced 24 h before analysis. Uptake was terminated by removing the medium and adding 500 μl of 0.1% Triton-X in PBS, separately to the specimens and to the original well. Radioactivity of the permeabilized cell suspension was determined with a β-scintillation counter (Beckman LS 3801, U.S.). Quenching was insignificant with up to 500 μl of 0.1% Triton-X used. Cell proliferation was also quantified using an MTS metabolic assay (CellTiter 96™ Aqueous, Promega Corporation, U.S.). At each time point, 500 μl of FBS-free culture medium and 100 μl of MTS reagent was added to both the specimen and the original well, to measure cell proliferation within the specimen and at the bottom of the original well. Plates were incubated for 2 h at 37°C, 5% CO_2. The absorbance of 5 aliquots per sample in 96 well plates was measured in a plate reader (Microplate reader, Anthos Labtec HT3) at a wavelength of 492 nm, and the mean of the five readings was taken for each time point. Background absorbance was corrected for by subtracting the absorbance index of culture medium from the specimen data. [^{3}H]-Thymidine labeling and MTS assay results represented the mean of 5 specimens. Statistical comparisons within each specimen group were conducted with a two-sample t-test, at 0.05 significance level. Correlation was established using standard Pearson's coefficient, r.

28.2.5 Histology and Immunocytochemistry

For histology and immunocytochemistry, specimens were embedded in tissue freezing medium (H-TFM, Triangular Biomedical Science, North Carolina) and fixed in liquid nitrogen in triplicates, at each time point. Frozen specimens were subsequently sectioned with a Leica CM3050 Cryostat Microtome. The 8-μm sections obtained were routinely stained with hematoxylin and eosin (H&E) and immunostained for collagen types I and III and fibronectin. The primary antibodies used were (1) Monoclonal mouse antihuman collagen type I, 1:100; (2) polyclonal rabbit antihuman collagen type III, 1:20; and (3) monoclonal mouse antihuman fibronectin, 1:100 (Chemicon, California). Blocking was performed with bovine serum albumin in PBS, for 30 min at room temperature prior to staining. Primary antibody staining was carried out in a wet chamber (sealed container with wet tissue paper) at 4°C overnight. Secondary antibody staining was performed with HRP-conjugated anti-mouse kit (DAKO Envision+, DAKO Corp., California) or HRP-conjugated anti-rabbit kit (Cell and Tissue Staining Kit, R&D Systems, Minnesota). Negative controls were similarly stained, replacing the primary antibodies with PBS. All slides were counterstained with hematoxylin, mounted, and viewed under an Olympus IX70 inverted light microscope, using a bright field filter.

28.3 RESULTS

28.3.1 Macroscopic Observations

Specimens were digitally photographed at each time point (Figure 28.3). Images showed that there was significant contraction of TissuFleece specimens. At week 1, the square TissuFleece specimens had contracted to spherical balls that were less than half the size of the original specimens. The contracted collagen spheres continue to shrink steadily and were only one-tenth of their original size at the end of week 3. There were no significant changes in size and appearance of the specimens in the other groups, although occasionally, Alloderm specimens became folded.

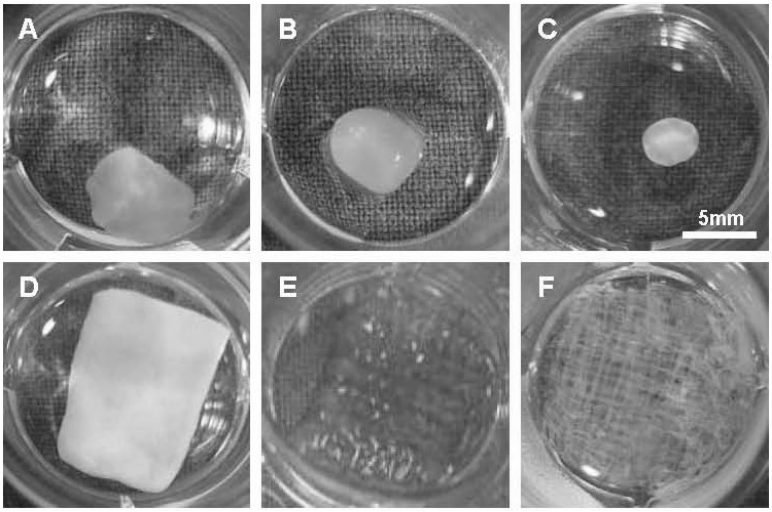

FIGURE 28.3 Gross morphology of four specimen groups during the *in vitro* culture period. TissuFleece specimens steadily decreased in size over weeks 1 (A), 2 (B), and 3 (C) in culture. Specimens contracted to less than half the original specimen size after 3 weeks. No change in specimen sizes of Alloderm (D), PLGA-PCL meshes (E), and chitosan scaffolds (F) was observed.

28.3.2 Cell Morphology and Viability

Cell morphology was analyzed with EM. At week 1, high vacuum SEM was used to view the specimens. The dehydration step in preparing the TissuFleece and Alloderm specimens for SEM resulted in the collapse of the specimens. Consequently, the image of the TissuFleece specimen at week 1 (Figure 28.4A) showed a low magnification of a shrunken specimen, while the Alloderm specimen surface appeared dry and rough (Figure 28.4B). On the other hand, the image of PLGA-PCL specimen at week 1 (Figure 28.5A) revealed that the cells have attached onto the fibers and were filling up the inter-fiber spaces within the knitted yarns. The inter-yarn pore spaces were unoccupied. Images of chitosan specimens at week 1 did not show significant cell attachment (Figure 28.5B). At week 2, ESEM was used to view all the specimens. The TissuFleece and Alloderm specimen surfaces appeared smoother than at week 1 (Figure 28.4C, D). Although cell-like structures could be observed on the TissuFleece specimen, it was difficult to ascertain the presence of cells on the surfaces of both these biological substrates based on ESEM images alone. Unseeded TissuFleece appeared to have a more fibrous surface topography compared to seeded ones (Figure 28.4G), while unseeded (Figure 28.4H) and cell-seeded Alloderm (Figure 28.4D, E) did not appear different.

On the PLGA-PCL specimens, the cells have proliferated along the fibers and appeared as a premature tissue-like formation within the inter-fiber pore spaces (Figure 28.5C). There were no differences in the chitosan specimens (Figure 28.5D). At week 3, the TissuFleece and Alloderm specimen surfaces did not appear to be significantly different from that at week 1 (Figure 28.4E, F). The premature tissue-like formation on the PLGA-PCL specimens had encapsulated the fibers and began to fill up the inter-yarn pore spaces (Figure 28.5E). There were again no observable differences in the chitosan specimens (Figure 28.5F).

Cell viability was analyzed with CLM. Viable cells could be observed clearly on TissuFleece, Alloderm, and PLGA-PCL specimens throughout the culture period, with typical spindle morphology (Figure 28.6). A proportion of nonviable cells were also present. Part of the red signals on Alloderm specimens came from the specimen itself, as revealed by non-cell-seeded controls. This was likely due to remnants of cell nuclei or DNA still residing within the acellular dermis (data

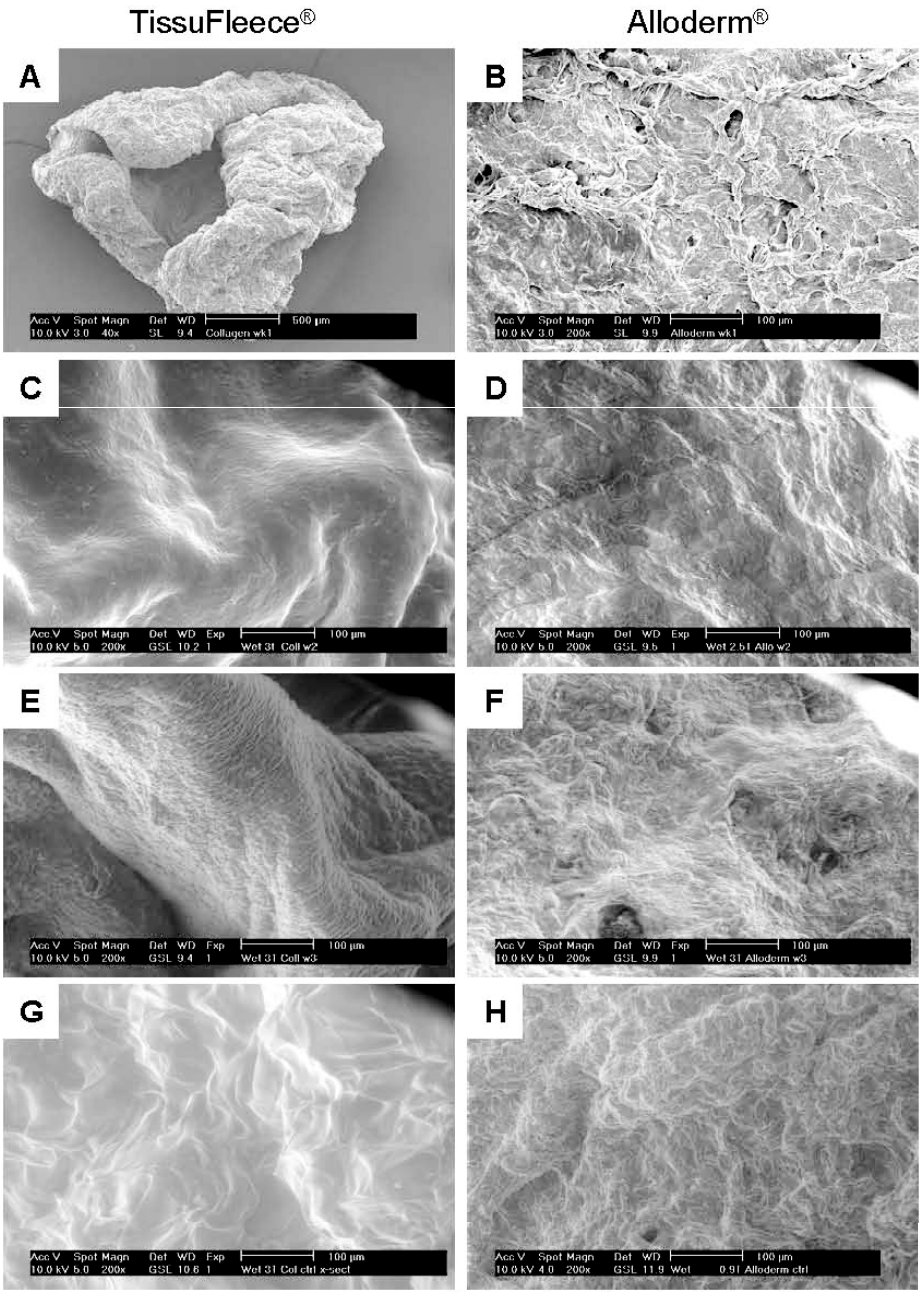

FIGURE 28.4 Representative EM images of TissuFleece and Alloderm specimens at week 1 (A, B), 2 (C, D), and 3 (E, F). SEM was used at week 1, resulting in collapsed specimen structure due to dehydration carried out prior to viewing. ESEM was used at weeks 2 and 3. However, it was difficult to ascertain the presence of cells on the surfaces of both these biological substrates based on ESEM images alone, even when compared to non-cell-seeded controls (G, H). Original magnification (A) ×40, (B–H) ×200.

not shown). In accordance with the SEM image presented, viable cells on the PLGA-PCL specimen were observed to align themselves along the fibers and proliferated within the inter-fiber space. Degrading PLGA fibers were stained red. Only a few viable cells were found on the chitosan specimen, and they did not express the typical spindle morphology of HDFs.

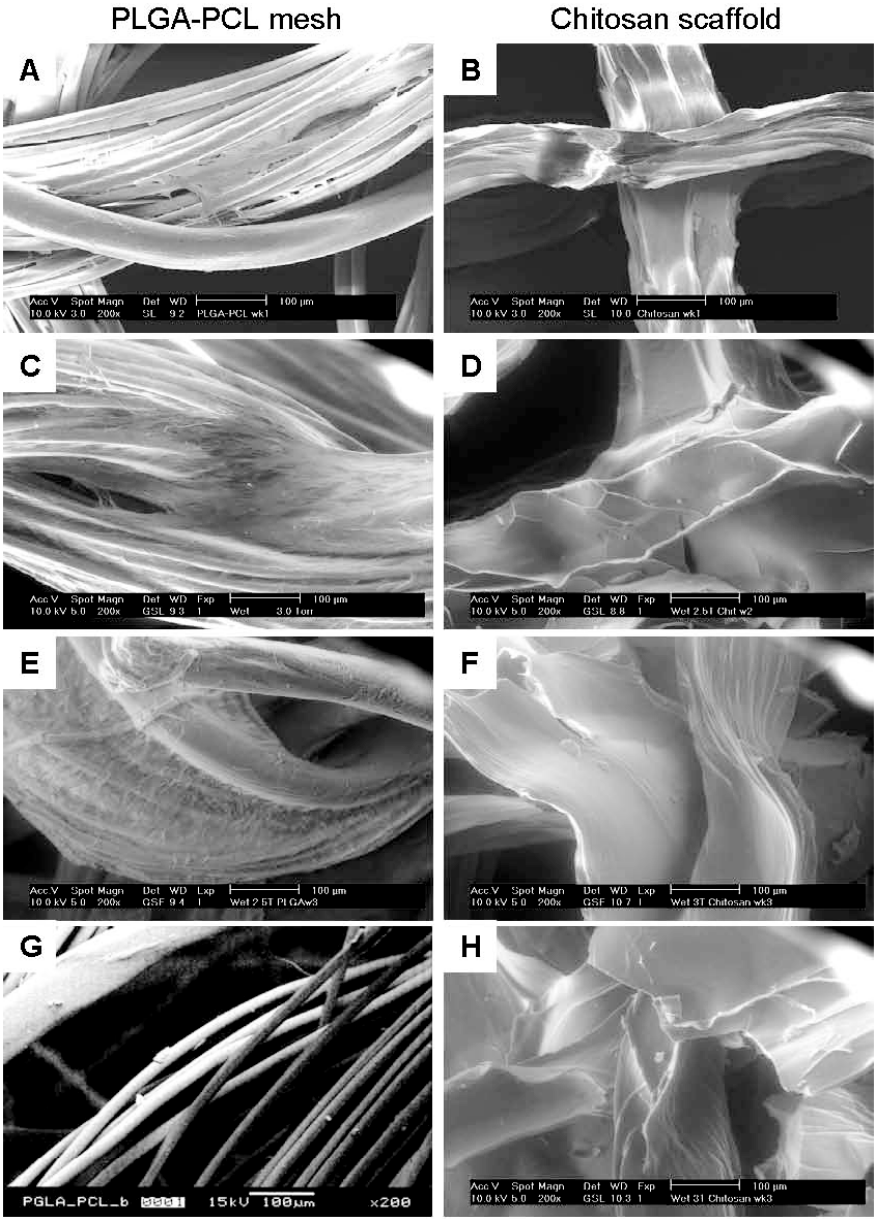

FIGURE 28.5 Representative EM images of PLGA-PCL and chitosan specimens at week 1 (A, B), 2 (C, D), and 3 (E, F). Both specimen groups did not collapse after dehydration, prior to SEM. A steady increase in cell density was observed in the PLGA-PCL meshes, resulting in a premature, tissue-like mass encapsulating the fibers and filling up the inter-yarn pore spaces at week 3. No observable tissue formation was detected in the chitosan scaffolds. (G, H) Non-cell-seeded controls. Original magnification (A–H) ×200.

28.3.3 Cell Proliferation

MTS assay and [^{3}H]-thymidine labeling results showed that metabolic activity and thymidine uptake patterns on the different substrates were significantly different. Figure 28.7A shows the MTS absorbance indices of the 3-D specimens while Figure 28.7B shows the absorbance indices of the 2-D culture plate used to house the specimens.

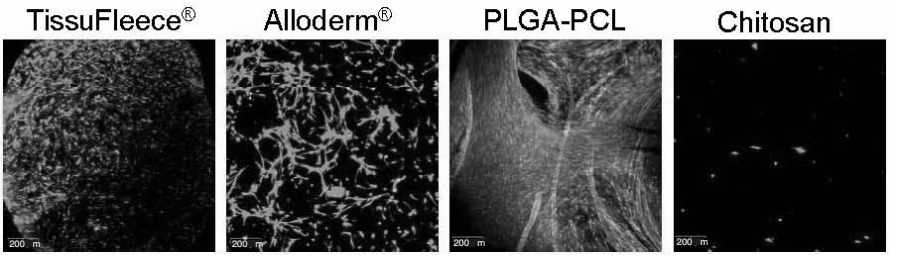

FIGURE 28.6 Representative CLM images of specimen groups, indicating cell viability at week 3 *in vitro* culture. Viable cells were stained green while nuclei of nonviable cells were stained red. Cells in the PLGA-PCL meshes proliferated and aligned themselves along the fibers, which fluoresce red as the material starts to degrade. Few viable cells were observed in the chitosan scaffolds. Original magnification ×100.

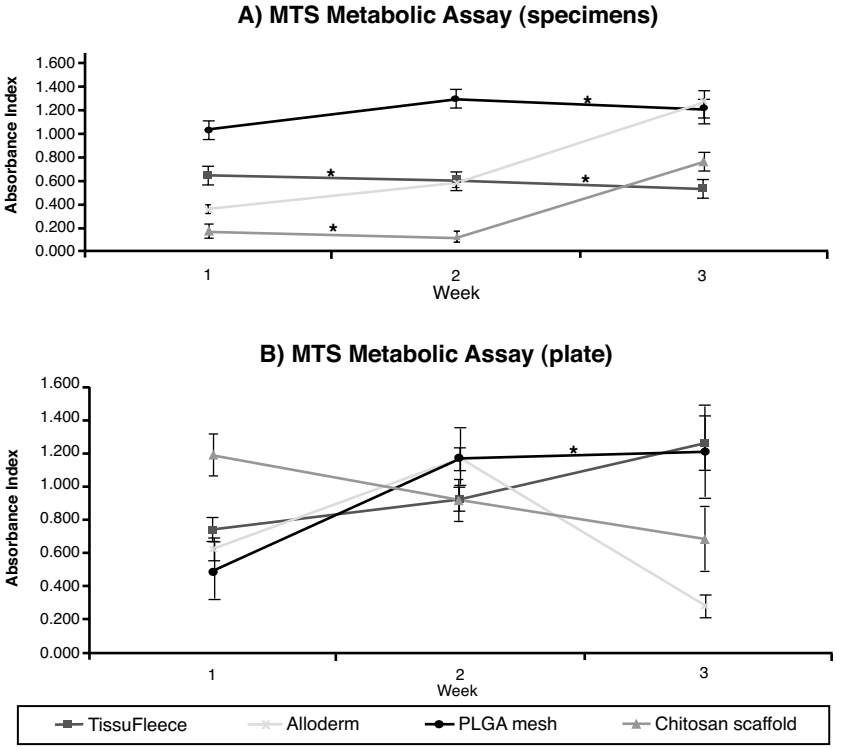

FIGURE 28.7 Results of MTS metabolic assay on the specimens (A) and on the bottom of the culture plate (B). Differing trends were recorded on different materials. Error bars represent ±standard deviation. * Statistically no difference ($p > 0.05$).

The absorbance indices for TissuFleece hovered around 0.5, with no significant variation ($p > 0.05$) over the culture period. On the other hand, the absorbance indices of the 2-D culture on the bottom of the plate increased by about 25% and 35%, respectively, from week 1 to 2 and from week 2 to 3. The absorbance indices for Alloderm specimens rose steadily over the three weeks, approximately doubling with each passing week. In the 2-D culture, the absorbance indices doubled from week 1 to 2, but dropped by 4 times from week 2 to 3. PLGA-PCL specimens recorded consistently high absorbance indices, increasing by about 20% from week 1 to 2 and showing no significant difference between weeks 2 and 3 ($p > 0.05$). A similar pattern was observed in the 2-D culture, where the absorbance index doubled from week 1 to 2 and then remained constant from

week 2 to 3 ($p > 0.05$). The absorbance indices for chitosan specimens remained low at weeks 1 and 2 ($p > 0.05$), but increased approximately fourfold at week 3. On the contrary, the 2-D cultures on the chitosan plate recorded a high absorbance index at week 1, but steadily declined to half the original value at week 3.

[^{3}H]-thymidine labeling results showed a differing trend from that in the MTS assay. Figure 28.8A shows counts per minute (CPM) for the 3-D specimens while Figure 28.8B shows CPM for the 2-D culture plates used to house the specimens. CPM for TissuFleece specimens peaked at week 1, but decreased by approximately 50% at week 2 and did not change significantly at week 3 ($p > 0.05$). On the culture plate, CPM dropped from week 1 to 2, but subsequently rose back to approximately the same level from week 2 to 3. Alloderm® specimens recorded fluctuating CPM values, decreasing sharply from week 1 to 2 and then approximately doubling again at week 3. A similar drop in CPM level was recorded on the culture plates for Alloderm specimens from week 1 to 2, which remained constant at week 3 ($p > 0.05$). CPM values of PLGA-PCL specimens remained comparatively low at weeks 1 and 2 ($p > 0.05$), but increased significantly by about 2.5 times from weeks 2 to 3. CPM values on the culture plates did not change significantly ($p > 0.05$) over the entire culture period. Similar to the trend with the MTS assay, chitosan specimens recorded low CPM values at weeks 1 and 2, but rose approximately 4 times from week 2 to 3. Also similar to the trend shown with the MTS assay, the 2-D cultures on the chitosan plate recorded a high absorbance index at week 1, but steadily declined to half the original value at week 3. Among the four specimen groups, only chitosan specimens showed strong correlation between MTS and [^{3}H]-thymidine labeling results ($r = 0.92$ on the specimens; $r = 0.79$ on the culture plate).

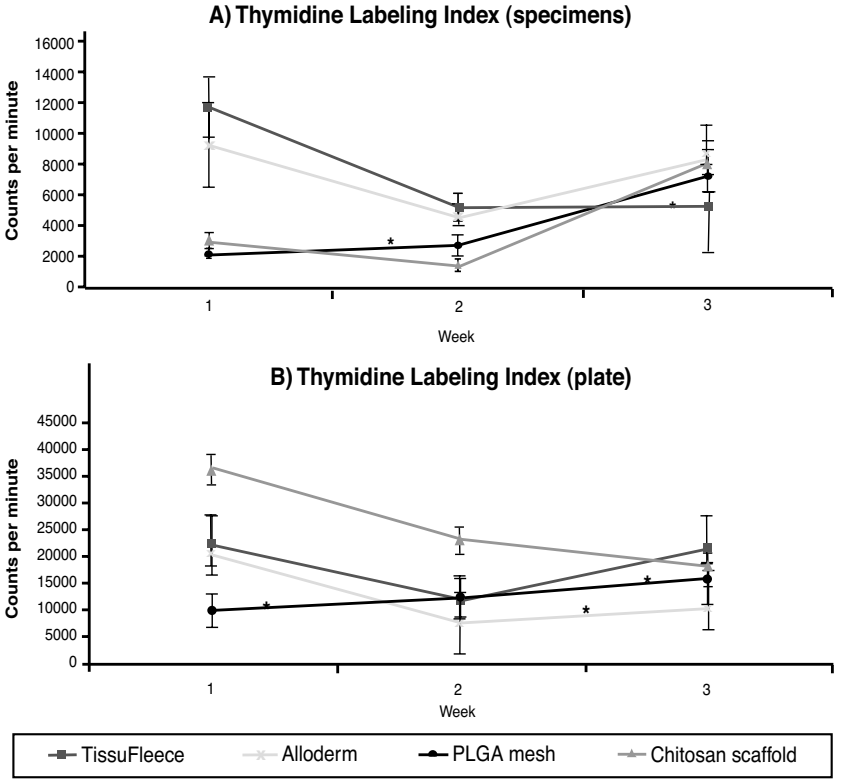

FIGURE 28.8 Results of thymidine labeling on the specimens (A) and on the bottom of culture plates (B). Differing trends were recorded on different materials. Error bars show ± standard deviation. * Statistically no difference ($p > 0.05$).

28.3.4 Histology

Histological results described here were representative of specimens within the same experimental group. H&E staining of cryo-sectioned specimens revealed different patterns of HDF proliferation within TissuFleece, Alloderm, and PLGA-PCL specimens. Figure 28.9 shows the histological images of TissuFleece and Alloderm specimens while Figure 28.10 shows that of PLGA-PCL and chitosan specimens. At week 1, a dense conglomeration of HDFs was observed near the surface of the TissuFleece (Figure 28.9A). Cell distribution over the specimen cross sections was highly inhomogeneous. Cells on Alloderm (Figure 28.9B) were also poorly distributed, proliferating only on the outer surfaces, with limited penetration into the dermal matrix. On PLGA-PCL, cell densities were low and much of the inter-fiber spaces were still empty (Figure 28.10A). Only fragments of chitosan scaffolds, which were stained red by eosin, remained on the glass slide after staining (Figure 28.10B, D, F), with almost no cells. At week 2, a dense conglomeration of HDFs was again observed in TissuFleece specimens (Figure 28.9C). Cells were still poorly distributed within each specimen. There was some penetration of cells into Alloderm specimen (Figure 28.9D) matrices at week 2. However, the majority of the cell population continued to proliferate around the specimen exterior. Premature tissue-like formation was observed within the inter-fiber spaces in PLGA-PCL specimens (Figure 28.10C). Cells formed a network which incorporated the PLGA and PCL fibers. Cells continued to proliferate as dense conglomerations within TissuFleece specimens at week 3, although a small proportion of cells were seen to penetrate into the surrounding material (Figure 28.9E). A greater proportion of cell penetration into the matrix was observed in Alloderm specimens. Figure 28.10F shows a folded Alloderm specimen with a dense population of cells in between the folded layers. Degradation of PLGA fibers could be observed at week 3, with the fibers shown in Figure 28.11E breaking up into multiple shorter segments. Although premature tissue formation was still observed within the inter-fiber spaces, cell proliferation into the inter-yarn spaces was restricted. From the controls, we could see that unseeded TissuFleece specimens (Figure 28.9G) had highly porous internal architecture while unseeded Alloderm specimens (Figure 28.9H) appeared denser, with extra-cellular matrix made up predominantly of collagen bundles. The unseeded PLGA-PCL and chitosan (Figure 28.10G, H) specimen sections were unstained because these sections were unable to be adhered onto poly-*L*-lysine coated glass slides. The sections showed well-defined circular cross sections of the PLGA and PCL fibers and the interconnected pore structure of chitosan specimens.

28.3.5 Immunocytochemistry

Representative images of immunocytochemistry results, at week 3 postseeding, are presented in Figure 28.11. Collagen types I and III and fibronectin were expressed in cell-seeded TissuFleece, Alloderm, and PLGA-PCL specimens. In TissuFleece specimens, ECM proteins were expressed in regions of dense cell clusters. Unseeded TissuFleece specimens did not stain positive for any of the ECM proteins (data not shown). In Alloderm specimens, ECM protein expression can be observed both at the peripheral of the specimens, where cells proliferated, and also within the matrix. Immunostaining on unseeded Alloderm confirmed that collagen types I and III and fibronectin were present in the native matrix (data not shown).

In the PLGA-PCL specimens, protein expression was more homogenous, as cells were better distributed within the inter-fiber space. Staining of unseeded PLGA-PCL mesh was not possible as sections did not adhere to the poly-*L*-lysine coated glass slides. However, the synthetic material did not stain positive in the seeded specimens, showing that there is no cross reactivity of the antibodies with the material. Sections of chitosan specimens did not adhere to the poly-*L*-lysine coated glass slides due to a lack of a critical mass of cells and ECM, and thus staining was not possible. Cell-seeded negative controls did not show any positive staining (Figure 28.11).

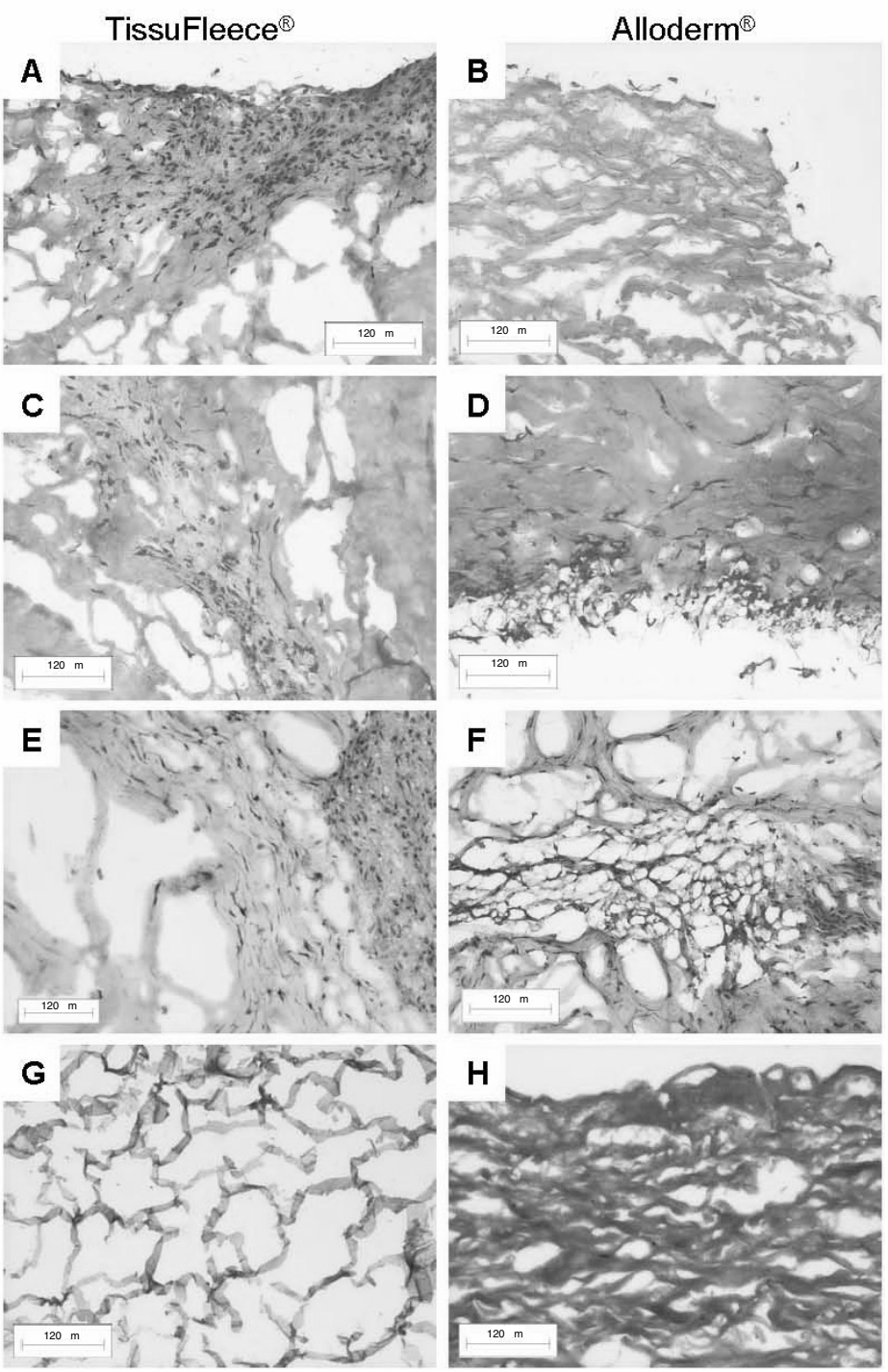

FIGURE 28.9 Representative histology images (H&E) of TissuFleece and Alloderm specimens, cell-seeded at week 1 (A, B), 2 (C, D), 3 (E, F), and nonseeded controls (G, H). Cell distribution was inhomogeneous in both specimen groups. Dense conglomerations were observed in the TissuFleece specimens while cells proliferated mostly on the exterior surfaces of Alloderm specimens. Original magnification ×100.

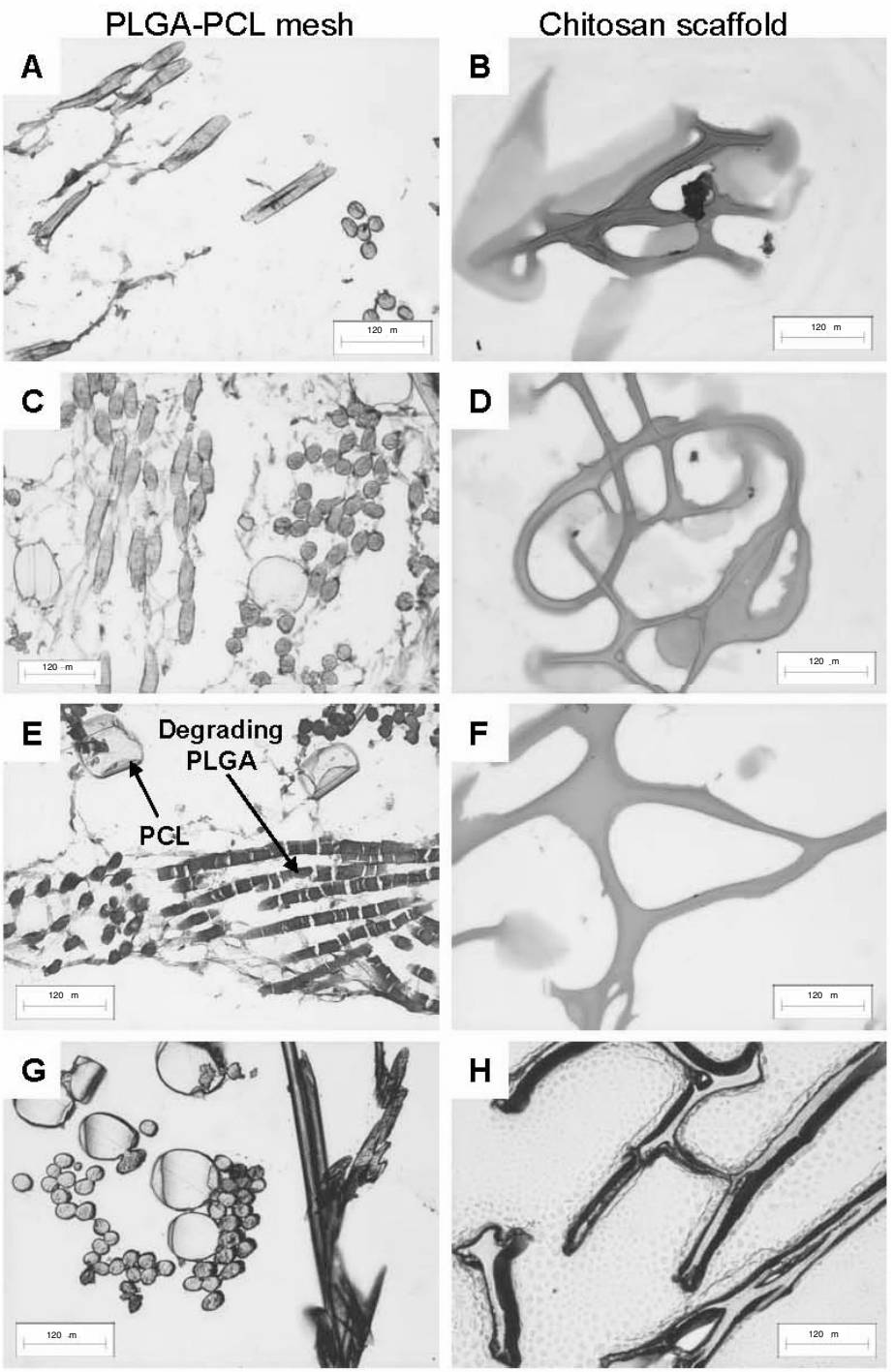

FIGURE 28.10 Representative histology images (H&E) of PLGA-PCL and chitosan specimens, cell-seeded at week 1 (A, B), 2 (C, D), and 3 (E, F), and nonseeded controls (G, H). Premature tissue formation was observed within the inter-fiber spaces of PLGA-PCL specimens from week 2, but cell proliferation into the inter-yarn spaces was restricted. Only fragments of chitosan specimens remained after staining, with few cells observed. Original magnification ×100.

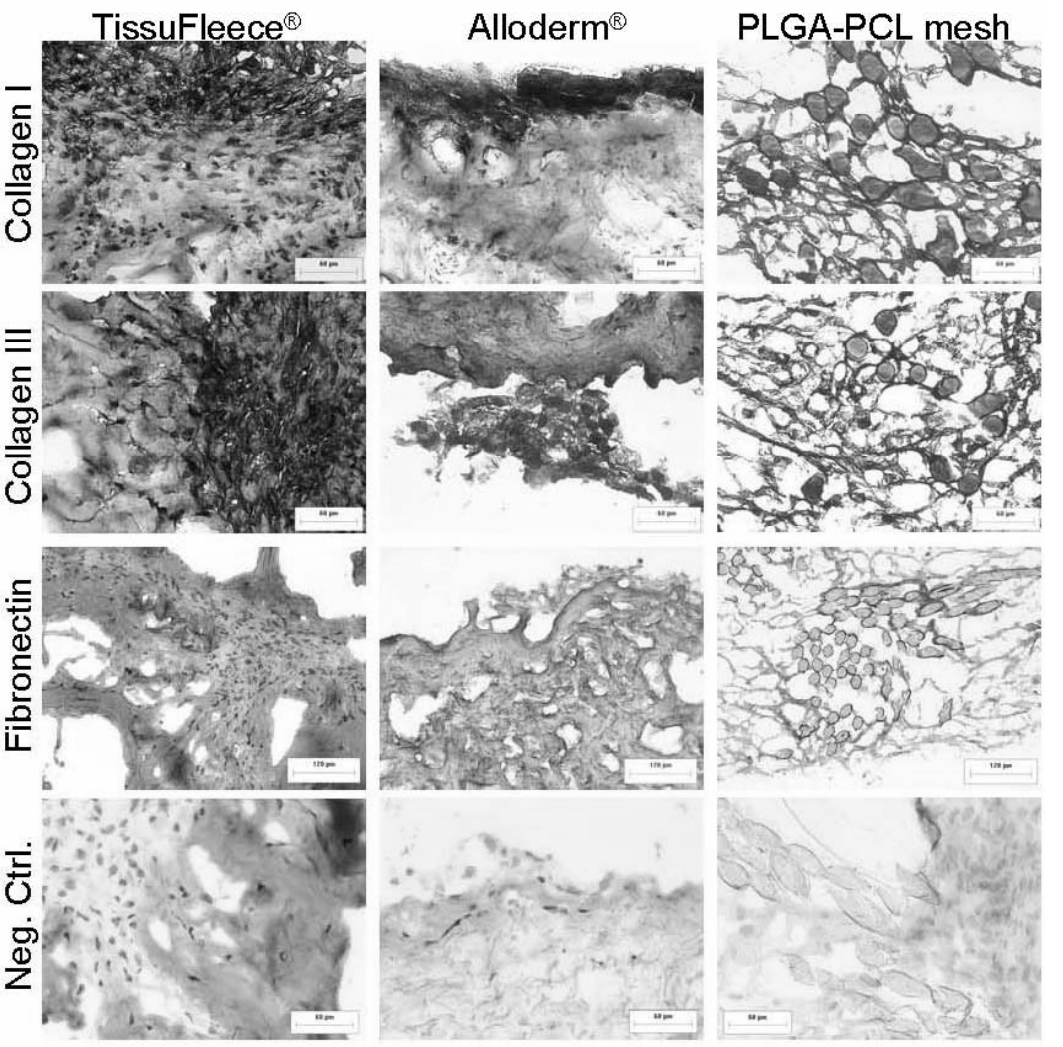

FIGURE 28.11 Immunostaining of specimen sections for collagen I and III and fibronectin, at the end of 3 weeks *in vitro* culture. All specimens showed healthy production of the ECM proteins. Original magnification: collagen I and III and negative control (×200); fibronectin (×100).

28.4 DISCUSSION

A major problem in culturing a dermal structure *ex novo* is the fact that cultured fibroblasts, in common with most cells, grow in two dimensions. For this reason, a three-dimensional dermal architecture cannot be achieved. To overcome this difficulty, three-dimensional structures have been used as supports for fibroblast growth, such as collagen-glycosaminoglycan matrices, allogeneic dermis, and synthetic polymers. Hence, in order to successfully produce a tissue-engineered skin equivalent as illustrated in Figure 28.1, optimal substrates for both the epidermal and dermal components must first be found. A potentially suitable epidermal substrate has been developed by the authors' group in the form of an ultra thin PCL film.[11,12,13] In parallel to the work being carried out on PCL films, a study was conducted to compare the suitability and efficacy of natural and synthetic matrices as dermal substrates.

28.4.1 Comparison of Scaffold Materials

28.4.1.1 TissuFleece

TissuFleece is a spongy foam made from equine collagen type I. The foam is highly porous, readily available in sterile package, and is being used clinically as a hemostatic dressing. It appears that such a material would be suitable as a substrate as the dermal component of a bilayered skin equivalent. Results from this study showed that TissuFleece was capable of supporting HDF attachment and proliferation, showing good cell viability, healthy morphology, and ECM protein expression. However, the foams contracted to approximately 10% of original size over the 3-week *in vitro* culture period. Fibroblasts in the tissue surrounding a wound site are known to secrete proteases to break down the provisional matrix and migrate into the wound site, where they actively proliferate, produce, and model new collagen to form the stroma or granulation tissue.[17] At the same time, fibroblasts in the granulation tissue differentiate into myofibroblasts,[18] which express smooth muscle actin and are responsible for the dermal contraction of wounds which brings about scarring.[19] In the same manner, HDFs in TissuFleece specimens could actively break down, remodel, and exert contraction forces on the collagen structure of the specimens, bringing about the marked reduction in specimen size observed. In addition, the poor interconnectivity of the TissuFleece pore structure brought about a poorly distributed cell population. Actual cell numbers in the specimens might not have changed significantly, based on the consistent metabolic activity level shown from the MTS assay. On the contrary, from [^{3}H]-thymidine labeling results, the proportion of cells in the S phase was high at week 1, but dropped at week 2 and remained constant at week 3. It can be hypothesized that as the collagen matrix was deformed via contraction, cells were induced to enter the quiescent G0 phase of the cell cycle, due to space constraint. A similar trend of decreasing DNA synthesis as collagen lattice contracted was also observed by Imaizumi et al.[20]

28.4.1.2 Alloderm

Alloderm specimens are dense acellular matrices of chemically and physically processed native human dermis, which proved capable of supporting healthy fibroblast growth *in vivo*. Wainwright[21] showed that Alloderm supported host cell infiltration and neovascularization when used as an acellular dermal graft in full-thickness burns. However, the dense matrix structure did not allow easy penetration of seeded cells based on the protocol of this *in vitro* study. As a result, cells remained close to the surfaces of the specimens, with some penetration at weeks 2 and 3, as seen from histological analysis. Similar results had been reported by others using cadaver deepidermalized dermis made in the same fashion as Alloderm.[22] The multiple cell layers on the surfaces of the specimens exerted contraction forces, resulting in the occasional folding of specimens, as shown in Figure 28.9F (Alloderm, week 3). A consistent rise in metabolic activity was observed in Alloderm specimens from week 1 through week 3. However, while the cells continued to proliferate and produce ECM, the proportion of dividing cells actually dropped from week 1 to 2, shown by the decrease in [^{3}H]-thymidine labeling index. The subsequent rise in [^{3}H]-thymidine uptake from week 2 to 3 was hypothesized to be the result of an increase in the number of cells entering the S phase as they began to penetrate and proliferate deeper into the collagen-based matrix.

28.4.1.3 PLGA-PCL

The use of bioresorbable synthetic materials such as the PLGA-PCL meshes eliminates the risks of pathogen transfer associated with the use of natural materials. Qualitative results from this study showed that PLGA (10:90) and PCL, used in the stated combination as a knitted mesh, was capable of supporting HDF growth, comparable to the natural materials used. The pattern of metabolic activity and [^{3}H]-thymidine uptake was, however, different from that observed in the TissuFleece and Alloderm specimens. Metabolic activity on the specimens and culture plates showed similar rising trends,

reaching a plateau at week 3, as premature tissue formation reaches equilibrium and contact inhibition on the 2-D culture surface set in. The rise in [^{3}H]-thymidine uptake on the specimens from week 2 to 3 could be an indication that a higher proportion of cells are proliferating into the inter-yarn spaces as inter-fiber spaces had become fully filled with premature tissue, as shown by CLM and EM images. A constant level of [^{3}H]-thymidine uptake on the culture plates of all four groups indicated a constant rate of proliferation of the cells in a 2-D environment. Taken collectively, the use of PLGA-PCL meshes gave the most satisfactory outcome. However, one disadvantage of the knitted PLGA-PCL meshes observed was that the inter-yarn space was too large. Cell proliferation into this space was limited over the 3 weeks in culture. The design of a denser yarn structure might help to alleviate this problem in future studies. The use of a cell carrier, such as fibrin, might not only ensure a higher seeding efficiency, but also enhance the homogeneity of the regenerated tissue.[2]

28.4.1.4 Chitosan

Chitosan had been shown to possess properties that may be beneficial in enhancing wound healing.[23] However, *in vitro* studies of chitosan with fibroblasts have produced opposing results. Howling et al.[24] compared the ability of 37%, 58%, and 89% deacetylated chitosan to modulate fibroblast mitogenesis *in vitro*. Results showed that highly deacetylated chitosan was generally more capable of stimulating fibroblast mitogenesis. However, this property was not universal for all fibroblast cultures, with varying results obtained with different donor cells, with no correlation with sex, age, or anatomical site. Studies by others have also showed both stimulatory[25] and inhibitory[26] effects. In this study, chitosan specimens did not appear to support HDF attachment and proliferation over 3 weeks. Images from EM, CLM, and histology did not reveal significant cell numbers or premature tissue formation. Few cells were detected and these lacked the spindle morphology expected of healthy HDFs. Both MTS assay and [^{3}H]-thymidine labeling showed a similar trend. The low level of metabolic and [^{3}H]-thymidine uptake at weeks 1 and 2 could be explained by the low number of cells present, as observed from the microscopy and histology images, which were largely dormant and not proliferating. A hypothesis for the unexpected rise at week 3 could be that any initially attached cells at this stage were adapting to the surface and material and becoming active, inducing the activation of cell cycle regulators, thus resulting in the significant rise in metabolic activity and [^{3}H]-thymidine uptake. A high level of metabolic and [^{3}H]-thymidine uptake on the culture plates at week 1 showed that most of the seeded cells did not attach onto the material but onto the plate bottom instead. These cells quickly reached confluence, accounting for the steady drop in absorbance and CPM indices over weeks 2 and 3.

28.4.1.5 Summary

A summary of the results from this study is presented in Table 28.1. In line with the objective of development of a bilayered skin graft, future studies will be conducted to assess the ability of these dermal equivalents to support the development of a stratified epidermal component.

28.4.2 Characterization Techniques and Data Interpretation

This study has demonstrated the need to use different characterization techniques in the analysis of different materials, of differing properties, as substrates for tissue regeneration. SEM and ESEM are commonly used by researchers in this field to assess and compare cell morphology on different substrates. In this study, SEM and ESEM did provide a good assessment of cellular morphology and surface topography of PLGA-PCL and chitosan specimens. However, these techniques were not suitable for materials that are unable to withstand the forces of contraction due to dehydration in sample processing and also while in the vacuum chamber of the microscope. In this study, TissuFleece and Alloderm specimens collapsed and shriveled in size while processing and also

TABLE 28.1
Qualitative Comparison of Various Properties between the Specimen Groups (+ null; ++ poor; +++ good; ++++ very good)

	TissuFleece	Alloderm	PLGA-PCL Mesh	Chitosan Scaffold
Ability to withstand contraction	++	+++	++++	++++
Cytocompatibility	++++	++++	++++	++
Cell distribution	++	++	+++	+
Matrix formation	+++	++	+++	+
ECM protein production	++++	++++	++++	+

during the actual viewing. Even with ESEM, wet samples began to dehydrate and lost their original morphology after an estimated 10 min inside the vacuum chamber, at a pressure setting of 3 Torr.

Care should be taken when interpreting data obtained from characterization techniques such as the MTS metabolic assay and [^{3}H]-thymidine labeling, both of which do not directly quantify cell numbers. The absorbance indices recorded using the MTS assay are direct measurements of metabolic activity of cells within the specimens, which might be correlated with actual cell numbers assuming a homogenous cell distribution in all specimens. The MTS tetrazolium compound (Owen's reagent) is converted by metabolically active cells into a colored formazan product that is soluble in tissue culture medium. The formazan absorbs at a characteristic wavelength, which is read on a standard ELISA plate reader.[27] Although the absorbance index obtained is proportional to the number of living cells, it does not provide any information on the relative proportions of metabolically active and inactive cells. Thus, a culture with a small number of metabolically active cells can give the same absorbance index as a culture with a large number of relatively inactive cells. Absorbance indices can only be correlated directly to cell numbers by comparing with a standard MTS absorbance calibration curve obtained with known cell numbers.

On the other hand, [^{3}H]-thymidine is incorporated into the DNA of cells undergoing DNA synthesis. The uptake of [^{3}H]-thymidine is thus an indication of the proportion of cells within the culture which are either in the S phase of the cell cycle, or undergoing DNA repair. Consequently, a culture with a small number of cells actively synthesizing DNA can show the same level of [^{3}H]-thymidine uptake as a culture with a large number of quiescent cells. Therefore, comparisons made between the different specimen groups or between different time points should be relative.

In contrast to this study, it has been reported in the literature that [^{3}H]-thymidine uptake generally correlates well with the MTS metabolic assay.[28–30] However, these studies had been performed on cells in 2-D monolayer cultures. In this study, strong correlation between metabolic activity and [^{3}H]-thymidine uptake patterns was only observed on the chitosan scaffolds ($r = 0.92$). In 3-D cultures, difficulties arise in ensuring complete penetration of [^{3}H]-thymidine and MTS reagent into the matrices to reach cells within, and subsequent complete extraction of the incorporated [^{3}H]-thymidine and reduced tetrazolium salt for assay. Further studies will be needed to optimize the use of such techniques in 3-D cultures. In addition, regardless of the cell seeding technique used, it is difficult to ensure an equal number of cells eventually seeded on scaffolds of different materials, due to variations in physical characteristics such as porosity and pore size. Comparisons of data between different specimen groups are problematic and should be treated with great caution.

28.5 CONCLUSION

TissuFleece, Alloderm, and knitted PLGA-PCL meshes were capable of supporting the regeneration of a dermal-like premature tissue *in vitro*, with healthy cell morphology, viability, and extracellular

matrix protein expression. However, the regenerated tissues in all three substrates were inhomogeneous. The patterns of metabolic activity and [^{3}H]-thymidine uptake were different between the specimen groups. TissuFleece contracted up to 90% of its original size over the culture period, with the formation of dense cell conglomerations. Cells were distributed over the surfaces of Alloderm specimens, with limited penetration into the matrix. Qualitatively, knitted PLGA-PCL meshes gave the best results in terms of their ability to support more homogenous cell distribution and withstand the cellular contraction forces. Metabolic activity was consistently high while a steady increase in [^{3}H]-thymidine uptake was also recorded. However, cells were unable to fill up the inter-yarn spaces completely. The chitosan scaffolds did not support dermal regeneration *in vitro*, based on this study protocol. These results demonstrated that besides cytocompatibility, physical characteristics, including porosity and ability to withstand cell contraction forces, play an important role in determining the optimal design of a scaffold for dermal skin tissue engineering.

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29 Biodegradable Polymers for Guided Nerve Regeneration

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CONTENTS

29.1 INTRODUCTION

The nervous system is a complex, sophisticated system that regulates and coordinates body activities. It has two major divisions[1]: central nervous system (CNS) and peripheral nervous system (PNS). The central nervous system consists of the brain and the spinal cord, while the peripheral nervous involves all the nerves that branch off from the spinal cord to the extremities. The fundamental cell of the brain is the neuron, which consists of a cell body, branch-like extensions off the cell body called dendrites, and at least one longer extension off the cell body called an axon. The dendrites conduct signals from their tips toward the neuron cell body whereas the axon carries message away from the cell body toward the terminal end of the axon. The neuron communicates with the other cells, such as effector cells, through the distal tips of the axon. Nerves are bundles of the axons from different neurons that carry signal in the same direction; nerves are essential intermediary connecting the brain to effector cells. Thus, if the nerves are severely injured, the signal between the cell body and the effector cells is interrupted, and neurons are unable to convey effective requests, such as a muscle movement.

Nervous system is not only a control mechanism in which thoughts, senses, and personality are being directed but is also responsible for carrying out the functions that support us to continue our lives and responding to external stimuli. Any possible defects and injuries within this system bring about termination of vital functions.

In general, the problems encountered in nervous system can be divided in two groups. In the first group, diseases occurred by faults of chemical structure within the nervous system and medication is applied for the treatment. Diseases that stem from problematic secretion of proteins, such as Alzheimer, Parkinson disease, Huntington, ALS, etc., can be counted in this group. Therefore, therapies have focused on correcting the defective gene (gene therapy), giving these proteins directly, or using controlled-release systems.

The second group in nervous system diseases consists of defects that occurred physically. These include death of nerve cells, axon degeneration, and myelin sheath damage.

Nerve conveyance is occurred by the means of electrical signals and neurons transmit these signals by axons. Axons are covered with myelin sheath. This sheath works as insulation material. Most common disease in this era is multiple sclerosis (MS).[2] The number of MS patients increased

recently. It is basically damage of myelin sheath. Stimulus directly crosses to another neuron not through synapse when this insulation lost. This misdirection causes losing of muscle control.

Injuries of peripheral nervous system generally affect axons. Deep cutoffs could reach nerve tissue and cause axon break-off. Axon regeneration occurs typically in four steps[3]:

1. Neuron turns on its initial state, axonal growth begins, and axons reach defected zone.
2. In this step, axons must pass over scar tissue.
3. Axons try to reach peripheral target.
4. After reaching target, reconstructing of transmission ways occurs.

This process is fairly slow. However, this mechanism cannot be completely successful. Oriented axon cannot continue its way for too long and aggregate in one point and forms tumor so called neuroma, which means the death of axon. Consequently, it is shown that functions of axons could not be regained completely after axon regeneration. Besides, axon regeneration is limited with peripheral nerve system. Neurons in spinal cord and brain have different characteristics and cannot regenerate themselves. Therefore, spinal cord damages bring about permanent insufficiency.[4,5]

29.2 NERVE REGENERATION AND MATERIALS FOR NERVE REGENERATION

A variety of methods have been proposed for peripheral nerve reconstruction, depending on how far the stumps are apart from each other. Direct suturing of the damaged nerve is possible, provided that the gap is small. If it is large, that is, larger than 3 cm for humans and 1–2 cm for rat, this approach is not desired because any tension introduced into the nerve cable would inhibit nerve regeneration. For regeneration of long gaps, nerve segments can be taken from the patient (autograft) or from a donor (allograft).[6,7] An autograft, which is the typical graft of choice, has disadvantages including a second surgical procedure, limited availability, and permanent denervation at the donor site. Allografts have also been commonly used, but these are accompanied by the usual need for immunosuppression and have very poor success rates.[8] Autologous and autogenous blood vessels and muscle fibers have also been used as conduits for nerve regeneration with varying levels of success, but these still suffer from some of the same disadvantages as autografts and allografts.[8]

To avoid the problems of the autografts and allografts, artificial nerve guidance channels have been developed. The nerve guide is a conduit that bridges the gap between the nerve stumps and directs and supports nerve regeneration. Several biomaterials have been used as nerve-guided channels, such as chitosan, laminin, fibronectin, and collagen, and synthetic materials including silicone elastomer, polyethylene, polyvinyl chloride, acrylic copolymer, and biodegradable polyesters.[9–15]

The biomaterials used as nerve guides should have some important properties. The material must be biocompatible, which means that it must be noncytotoxic, noncarcinogenic, nonimmunogenic, and nonmutagenic and must cause no irritation or allergic response, either local or systemic.[1] Degradation rate of the nerve guide is the other important parameter. It should be in accordance with the axonal growth rates. For instance, based on information obtained from a silicone-chamber model, a biodegradable graft for a 10-mm gap in the rat static nerve should maintain its strength for 8 weeks or longer in order to ensure that axons have entered the distal stump and been myelinated.[10] Furthermore, a nerve guide should be flexible, permeable or semipermeable, and easily handled for surgery. It might be transparent, which can allow accurate observation of the nerve stumps when telescoping them into the nerve guides.[11]

Transport properties of the nerve guide channels can influence the regeneration process by modulating solute exchange between the regenerating and extrachannel environments. Aebischer et al.[16] have reported that perm-selective channels with a Mw (molecular weight by weight) cutoff of 50,000 Da allowed the regeneration of the nerves, which more closely resemble the normal static nerve than channels that are impermeable or freely permeable to water solutes. They have hypoth-

esized that controlled exchange across the nerve guide wall enhances the formation of an optimal regenerating environment in addition to providing axonal guidance, preventing scar tissue invasion, minimizing the escape of growth factors released by the nerve stumps, and preventing the release of nerve antigenic factors.

Nerve grafts can be combined of two layers, of the same or different materials, to obtain the better transport and retention properties. The inner surface of the tube is usually a thin, nonporous membrane in order to act as a primary permeability barrier and serves to retain neurotrophic factors. The external layer, which is thicker and porous, provides structural support.

Silicone is the most popular material for guided nerve regeneration. Silicone tubulization of the nerve gaps has been the standard experimental model for many years[17,18] and has been shown to improve regeneration compared with defects that were not contained within a tube. However, in clinical use the silicone tubes have presented problems for long-term recovery. Silicone tubes typically have become encapsulated with fibrous tissue, and this has led to constriction of the nerve, necessitating a surgical procedure to remove the tube.[11] For instance, Merle et al.[19] reported successful nerve regeneration after reconstruction of the peripheral nerves in three patients, with the silicone nerve guide. After 2 years, the patients began complaining about secondary nerve impairment and irritation at the implantation site. Silicone guides were removed by means of secondary operation that might damage the regenerated nerves. Because of these disadvantages of silicone and other nonresorbable nerve guides, natural and synthetic biodegradable nerve guides have been developed.

Most commonly used natural materials in nerve regeneration are collagen,[13] chitosan,[20] alginate,[21] and their derivatives.[12]

Chitosan, the fully or partially deacetylated form of chitin, is the most extensively used material derived from chitin. It has been reported to possess excellent biocompatibility. Jianchun et al.[20] have reported that neurons cultured on the chitosan membrane can grow well and the chitosan conduit can greatly promote the repair of the peripheral nervous system. They have also studied the nerve cell affinity of the chitosan-derived materials, including glutaraldehyde-crosslinked chitosan, glutaraldehyde-crosslinked chitosan–gelatin conjugate, a chitosan–gelatin mixture, and a chitosan–polylysine mixture. It has been indicated that both chitosan coated with polylysine and the chitosan–polylysine mixture have excellent nerve cell affinity, defined as the ability to promote nerve cell to growth and function normally.[22] Chitosan can be easily formed as a nerve conduit due to its film-forming properties. However, chitosan has some disadvantages in use as a nerve guide. It is, however, more rigid and brittle than nerve tissues. Modulus of the swollen chitosan film is about 6 MPa when young modulus of spinal cord tissue is about 0.2–0.8 MPa. Moreover, swelling properties of chitosan can cause some problems, such as compressing regenerating nerve cells, *in vivo*. Cheng et al.[12] have been proposed to use chitosan–gelatin blend for peripheral nerve regeneration. All chitosan–gelatin films in different blend ratios have showed better nerve cell affinity than chitosan film. The composite film with 60-wt% gelatin has shown the best mechanical and cell affinity properties.[12]

The chitosan tubes derived from crab tendons have a naturally tubular structure, the hollow nature of which is also suitable for nerve regeneration.[23] However, they have the same problems as chitosan nerve-guided tubes. Their mechanical strength is very low for a lateral direction, though it is high for a long axial direction. Moreover, the tube walls swell to reduce the inner space of the tubes *in vivo*. These properties can cause to squash when the chitosan tubes are implanted *in vivo*. Recently, chitosan–apatite composites have been developed in order to try to solve these problems.[23] In animal studies using rats, chitosan–apatite tubes have demonstrated preferable biodegradation and biocompatibility and have regenerated nerve tissue well in the hollows of the tubes.

Collagen is one of the major components of the extracellular matrix. It has been used as material in various surgical prosthesis and thus in nerve repair.[24,25] It is principally biodegradable and depending on the application, its resorption rate can be adjusted by different crosslinking methods.[26] In nerve regeneration applications, collagen plays a dual role, serving as a physical framework for

regeneration nerves[27] and as a source of various trophic factors for regenerating axons, especially in early regeneration.[28] Keilhoff et al.[29] have tested Schwann cell implanted collagen type I/III tubes in rats as a potential nerve-guiding matrix. The collagen conduits have been completely revascularized between day 5 and 7 postoperatively and well integrated into the host tissue. They reported that implanted Schwann cells adhered, survived, and proliferated on the inner surface of the matrix and formed nerve-guiding columns of Büngner. Itoh et al.[13] have investigated the effect of crosslinking method of collagen tubes on peripheral nerve repair. They have tested collagen tubes crosslinked by three different methods, namely irradiation by UV, heating, and immersing in glutaraldehyde. *In vivo* studies in rats have shown that UV irradiation crosslinking is the best method for collagen nerve conduits. It has been also found that the regenerating axons and migration of Schwann cells were affected by such physical changes of the nerve guide tubes. In another study, Chamberlain et al.[24] have studied the effects of the tube composition and presence of a specific glycosaminoglycan (CG) substrate on the properties of nerve regenerated over 60 weeks across a 10-mm gap in the rat static nerve. They have reported that large-pore collagen tubes with the CG matrix significantly increased the number of axons per nerve compared with either unfilled or small-pore collagen tubes.

Collagen nerve guides have also been tested by filling them with magnetically aligned type I collagen gel.[30] Recently, Yoshii et al.[31] have developed a nerve guide made of collagen filaments, instead of a tube, to improve resorbability and permeability of the material employed and assessed its effect in peripheral nerve regeneration.

Alginate is a bioresorbable long-chain polysaccharide extracted from brown seaweed. It has been examined in spinal cord repair in the rats.[14] Alginate gel covered by polyglycolic acid mesh tubes have been used to regenerate cat static nerve.[32] Nerve regeneration has occurred through the guided tube. Besides these, alginate gel without tubulation has also been used as an artificial nerve guide to repair a 7-mm gap in peripheral nerves.[21] It has been observed that nerve fibers could have been regenerated through the alginate gel and repopulated the distal nerve stump.

Although they have been shown to improve the biocompatibility, decrease the toxic effects, and enhance the migration of support cells (such as Schwann cells), there are some difficulties in the use of natural materials. They may cause undesirable immune responses and appear to have the potential for immunosuppression and batch-to-batch variation in large-scale isolation procedures.[33] Because of the problems in use of natural materials, synthetic materials have also been employed. Variations in the chemical and physical properties of synthetic materials allow obtaining the materials in different geometric configurations, porosity, degradation rate, and mechanical strength. The variation in these parameters can dramatically affect the ability of axons to proliferate.[34–36]

Polylactic acid, polyglycolic acid and its copolymers, poly(*L*-lactide-co-caprolactone), poly(phosphoester), and polyhydroxybutyrate have been used as a conduit for guided nerve regeneration. Grijpma et al.[37] tested a guide made of semicrystalline copolymer of *L*-lactide and ε-caprolactone (50/50 mol/mol). Although nerve regeneration through this guide was good, fragments of the material surrounding the regenerating nerve were still present 2 years after implantation. These fragments can cause chronic irritation at the implantation site and scar tissue formation, in turn leading to constriction of the nerve and therefore negatively influence nerve function in the long term.[38] To avoid this problem, Den Dunnen et al.[39] have developed a new biodegradable nerve guide from a copolymer of poly(*D,L*-lactic acid) and poly(ε-caprolactone) (1:1, D:L = 15:85). Due to the lower crystallinity of *D,L*-lactide, these new regeneration tubes have shown faster degradation and degraded completely within 1 year. Moreover, they reported that the poly(DLLA-ε-CL) nerve guide showed fast nerve regeneration across a 10-mm gap in the static nerve of the rat,[34] even faster and qualitatively better compared with regeneration through a 7-mm autologous nerve graft.[40] Rodriguez et al.[41] also discussed nerve regeneration ability of polylactide/caprolactone nerve guides with different degrees of permeability by comparing to permanent guides of polysulfone (POS). It has been observed that highly permeable polylactide/caprolactone guides allowed for faster and higher levels of regeneration than impermeable or low-permeable polylactide/caprolactone guide, while semipermeable POS guides showed very low level of regeneration. Laminin-coated poly(*L*-

lactide) filaments have been examined for suitability in supporting directional growth of Schwann cells and axons using dorsal root ganglia *in vitro*.[42] Results have shown that nerve attachment and growth have been induced when the PLA filament was coated by laminin. Implants designed by arranging these filaments into bundles may be used for axon regeneration to provide guidance for nerve cells.

Matsumoto et al.[43] have designed a new nerve conduit composed of a biodegradable tube filled with biodegradable filaments. The tube was made of cylindrically woven PGA mesh coated with amorphous collagen in both surfaces. This tube has been filled with laminin-coated collagen fibers. This developed matrix has induced axonal regeneration over an 80-mm nerve gap in dogs. Due to the difficulties in manufacturing of fine collagen fibers, the same nerve conduit has been tested by filling with collagen sponge.[44] This new matrix promoted axonal regeneration over an 80-mm nerve gap with the same efficacy as the same conduit filled with collagen fibers.

Poly-3-hydroxybutyrate (PHB) has also been tried as a nerve conduit. PHB is a storage product of bacteria, occurring within the cell cytoplasm as granules.[45] It degrades hydrolytically and can be absorbed in 24–30 months *in vivo*. Nerve conduits made of PHB have shown good axonal regeneration with a low level of inflammatory infiltration in rats.[46] In another study, biodegradable nerve guides made of poly(glycolide-co-[ε-caprolactone]) and PHB polymer system have been examined in the rats.[15] This polymer system can be easily formed as a nerve tube. Moreover, this material offers several advantages, such as very low degree of swelling, good elastomeric properties, and desired degradation rate.

29.3 CONCLUSIONS

Although several groups have developed biodegradable natural and synthetic polymer-based nerve guides, there is still much work to be done to design a nerve guide that will provide nerve cell attachment and growth and that has the desired mechanical, geometrical, and permeability properties for guided nerve regeneration. In fact, a lot of research is still required to develop therapeutic strategies that might be useful in the complex of nerve regeneration.

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