MICRO AND NANOTECHNOLOGIES IN ENGINEERING STEM CELLS AND TISSUES

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PREFACE

More than a million people worldwide are in need of an organ transplant while only 100,000 transplants are performed each year. More than 100,000 Americans need a transplant each year but only 25,000 transplants are performed. Tissue engineering has become increasingly important as an unlimited source of bioengineered tissues to replace diseased organs. Tissue engineering attempts to build body parts by assembling from the basic components of biological tissues, namely, the matrix, cells, and tissue morphogenetic growth factors. As tissue-specific cells are limited in quantity, stem cells with their ability for self-renewal and pluripotency are becoming increasingly important as a cell source in regenerative medicine. These cell sources include but are not limited to bone marrow–derived stromal cells and hematopoietic cells, umbilical cord–derived stem cells, and induced pluripotent stem cells. Top-down approaches utilizing porous scaffolds with random or well-defined pore structures, seeded with cells and growth factors, have been used, in some cases successfully, as cellular constructs in the clinically relevant length scale in regenerative medicine. However, top-down approaches cannot recreate the intricate structural characteristics of native tissues at multiple nano- and microscales, leading to the formation of less than optimal composition and distribution of the extracellular matrix. It should be emphasized that the hierarchical organization of native biological tissues is optimized by evolution to balance strength, cell–cell and cell–matrix interactions, growth factor presentation, and transport of nutrients. Consequently, bottom-up approaches to build a single modular unit to mimic the structural features of native tissues and to serve as a building block for assembly to a larger tissue scale have received more attention in recent years. The processes of cell adhesion, migration, differentiation, extracellular matrix formation, and cell maturation depend on interactions at multiple length scales between the cell surface receptors

and their corresponding ligands in the matrix. The success of engineered tissues as an unlimited source for replacement of damaged organs depends on our depth of understanding of those interactions and our ability to mimic those interactions using enabling nano- and microscale technologies and to build modular scalable units for implantation. This book provides an overview of enabling micro- and nanoscale technologies in designing novel materials to elucidate the complex cell–cell and cell–matrix interactions, leading to engineered stem cells and tissues for applications in regenerative medicine. The editors, Murugan Ramalingam, Esmaiel Jabbari, Seeram Ramakrishna, and Ali Khademhosseini, thank the authors for their contribution to this timely book.

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STEM CELLS AND NANOTECHNOLOGY IN TISSUE ENGINEERING AND REGENERATIVE MEDICINE

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1.1 A BRIEF HISTORY OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Awareness of the natural regenerative capabilities of the human body dates back to ancient times. In Greek mythology, when Prometheus stole fire from the gods and gave it to the mortals, Zeus punished him by tying him to a rock and having an eagle peck away his liver, only to have it regrow and be eaten again the following day. Although the liver has significant natural regenerative capacity that seems to have been apparent for many ages, many other organs have a very limited ability to regrow after damage or removal. These limitations have spurred the development of regenerative approaches in the history of modern medicine, as clinicians and scientists continuously attempt to overcome the body's natural limitations.

The birth of whole-organ transplantation techniques has paved the way for modern developments in regenerative medicine and tissue engineering. Alexis Carrel, winner of the Nobel Prize in Physiology or Medicine in 1912 and the father of whole-organ transplant, was the first to develop a successful technique for end-to-end arteriovenous anastomosis in transplantation. In the 1930s, assisted by Charles

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Lindbergh, he developed a "perfusion pump" that allowed organs to be maintained outside the body during transplantation, a concept that has been more recently used in bioreactors for tissue engineering studies.[1,2]

The limitations of organ transplant because of immune reaction were recognized early. Gibson and Medawar found that application of a second skin allograft from the same donor resulted in faster rejection than the first, suggesting that the response may be immunologic.[3] Additional studies in dogs showed that allografts produced a mononuclear reaction to the transplanted organ.[4] Thus, to avoid the immune response, the first successful kidney transplant was performed in 1954 between identical twins.[5] It was not until the development of immunosuppressive drugs that transplantation from genetically different donors became feasible. Many advances have been made in the field of organ transplantation, including the development of immunomodulating therapies and significant reductions in the number of immunosuppressive drugs necessary after transplantation.[6,7] However, limitations because of organ or tissue availability and the continual need for chronic immunosuppression remain and have left physicians and scientists looking for a new approach that mitigates these issues. These efforts have resulted in the popularization of the fields of tissue engineering and regenerative medicine.

A succinct definition of regenerative medicine has been provided by Mason et al., stating that "regenerative medicine replaces or regenerates human cells, tissue or organs, to restore or establish normal function."[8] This broad definition can include the use of cell-based therapy, gene therapy, nonbiological devices, and tissue engineering strategies. Organ transplantation falls short in this definition because completely normal function is not possible given the need for continuous immune suppression.

Although the terms *tissue engineering* and *regenerative medicine* are sometimes used interchangeably, it is important to understand that tissue engineering falls under the umbrella of the regenerative medicine field but is not all encompassing. As defined by Langer and Vacanti, 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."[9] More specifically, tissue engineering uses a combination of cells, scaffolds, and bioactive factors in strategic combinations to direct the *in vitro* formation of new tissues or organs (Fig. 1.1). Regenerative medicine strategies, on the other hand, often rely on the body's natural processes to assist in the formation of new tissues after delivery of exogenous cells, scaffolds, or biomolecules.

As scientists have begun to unravel the complexity of biological processes on a cellular and molecular scale, the fields of tissue engineering and regenerative medicine have migrated toward applying this knowledge to control the interactions between cells and their environment, whether natural or assisted. Concomitantly, the applications of nanotechnology to biological processes have rapidly increased in recent years and hold the great promise for successful translation of bench research into the clinic.

In this introductory chapter, we will provide an overview of the central concepts in regenerative medicine and tissue engineering strategies. First, we will provide a

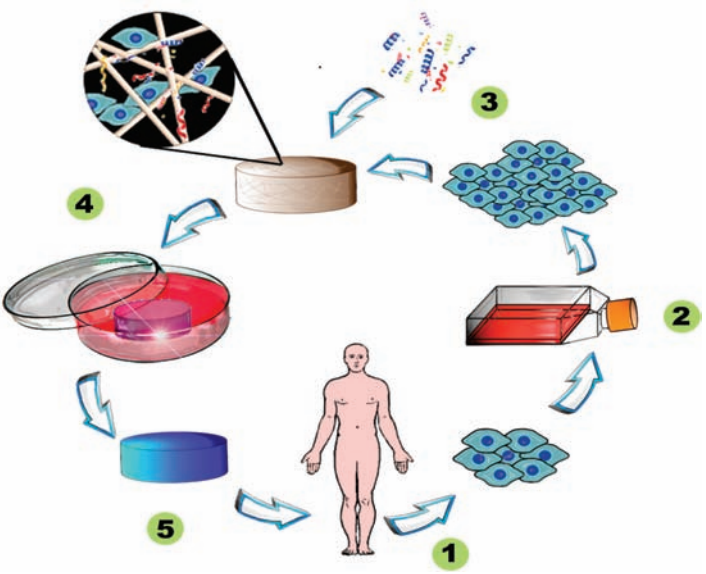

FIGURE 1.1 General schematic of tissue engineering strategy. (1) Cells are isolated from the patient and (2) expanded in 2D culture. (3) Expanded cells are then combined with various natural or engineered bioactive molecules (e.g., growth factors, nanoparticles, or DNA) into biocompatible scaffolds and (4) cultured *in vitro* under specific culture conditions to promote tissue formation. (5) Finally, functional tissue-engineered constructs are implanted into the donor to replace the damaged tissue.

brief overview of stem cells and their role in cell therapy strategies followed by a discussion of tissue engineering applications using stem cells in conjunction with biomaterials and bioactive factors. In particular, we will focus on the shift that has occurred in the field toward using nanoscale approaches that control cellular activities and tissue formation at the subcellular level.

1.2 INTRODUCTION TO STEM CELLS

To eliminate the need for immunosuppression, regenerative medicine and tissue engineering approaches have generally focused on using donor-derived autologous cells or cells that will not elicit an immune response. However, terminally differentiated cells are typically limited in their ability to proliferate, and it is therefore difficult to obtain sufficient cell number for regeneration of tissues. In addition, cells from tissues to be treated or replaced are likely to have undesirable defects that require structural repair. Because of this limited potential using differentiated cells, the use of stem or progenitor cells has become ubiquitous in the fields of tissue engineering and regenerative medicine. The characteristics that define stem cells—capacity for self-renewal, long-term proliferation, and the ability to differentiate into

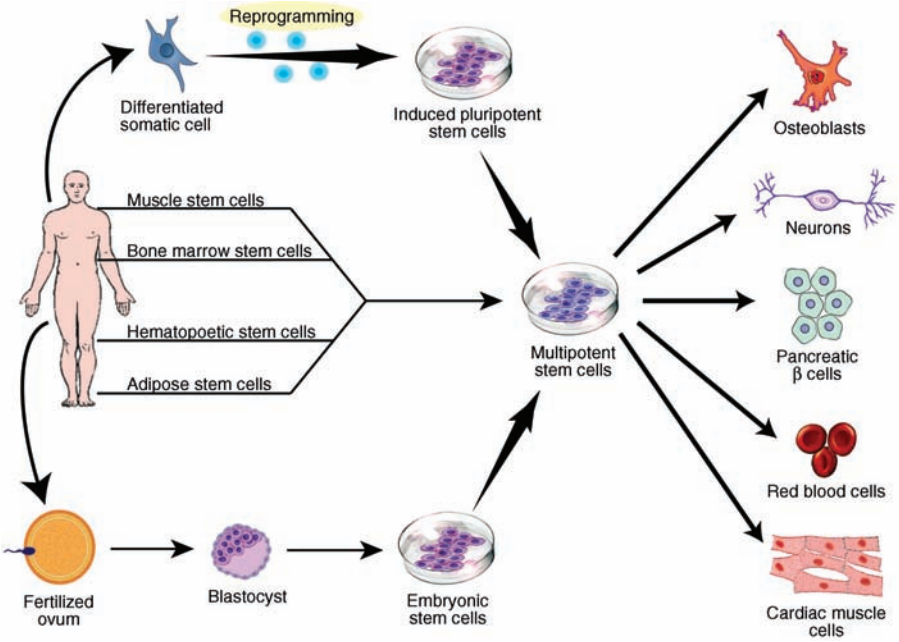

FIGURE 1.2 Isolation and differentiation of stem cells for tissue engineering and regenerative medicine. Multipotent stem cells can be obtained directly from various human tissues, including, but not limited to, bone marrow, blood, muscle, and adipose tissue. Pluripotent stem cells can be derived from the inner cell mass of the blastocyst (embryonic stem cells) or by reprogramming cells that were previously differentiated into a pluripotent state using specific factors (induced pluripotent stem cells). Multipotent stem cells can then be differentiated into many different cell types, only a few of which are shown here.

several different cell types—make them the optimal cell source for the development of new tissues and organs.

Stem cells are typically classified into two main groups: embryonic and adult. Figure 1.2 provides a summary of the origin and differentiation capacity of the different types of stem cells. Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocysts in the developing embryo before implantation. These cells are pluripotent, that is, they have the ability to differentiate into cells comprising all three germ layers and can be maintained in culture in an undifferentiated state for indefinite periods of time.[10] Although these characteristics make ESCs an attractive cell source for regeneration of tissues and organs, they have several limitations. First, ESCs present an ethical issue because embryos are destroyed in the process of obtaining the cells; second, because they are obtained from allogeneic sources, there is the possibility of an immune response, although research suggests that this response may be weaker than with traditional organ transplant;[11,12] third, because of their undifferentiated state, there is a possibility that ESCs can become tumorigenic and malignant.[13]

Adult stem cells, on the other hand, are isolated from postnatal tissues. They can also differentiate down multiple lineages but are more restricted in the types of cells that they can become and have more limited proliferation potential in comparison to ESCs. One advantage that adult stem cells have over ESCs is that they can often be obtained from the patients themselves, thereby eliminating the issue of immune rejection. However, if the tissue that contains the desired cells is diseased or has limited availability of stem cells (e.g., nervous tissue), it may not be possible to obtain autologous cells. Adult stem cells have been isolated from many different tissues, but one of the most widely used cell sources is bone marrow-derived mesenchymal stem cells (MSCs). These cells have been extensively characterized, and large numbers can be obtained with relative ease through a bone marrow biopsy. MSCs have the ability to differentiate into cartilage, bone, and fat, making them particularly useful for regeneration of musculoskeletal and connective tissues. In addition to their multipotent differentiation ability, MSCs have been shown to be hypoimmunogenic as well as immunosuppressive even after differentiation.[14,15]

Although adult stem cells are multipotent, unlike ESCs, they are somewhat lineage-restricted and typically only differentiate readily toward a few cell types and therefore are useful for a limited number of tissues. Recent advances, however, have led to the ability to reprogram somatic cells, including those that have undergone lineage specific differentiation, into an ESC-like state. Takahashi et al. showed that the retroviral-mediated expression of four nuclear transcription factors—Oct4, Sox2, Klf4, and c-Myc—resulted in cells with expression patterns and differentiation capacity cells similar to those of ESCs. These induced pluripotent stem cells (iPS cells) may allow scientists to overcome the ethical and immune rejection concerns of ESCs while retaining the increased proliferation and differentiation capacity that make the use of ESCs over typical adult stem cells desirable. Initial concerns that using viruses or vectors that integrate into the genome may lead to the development of tumors are being addressed by using transient or removable vectors or by directly delivering proteins.[16–21] Despite being isolated from the same individual, recent studies show that iPS cells may generate an immune response upon implantation,[22] suggesting that although these cells may have promise, a great deal more research must be done before using them in a clinical setting.

1.3 TISSUE ENGINEERING AND REGENERATIVE MEDICINE STRATEGIES

1.3.1 Cell Therapy

With the rising popularity of stem cell research, cell therapy has evolved as a potential treatment method for a variety of conditions. Cell therapy involves delivery of cells either into the bloodstream or directly into the tissue of interest. Although tissue engineering strategies combine cells with scaffold materials and bioactive factors before implantation, cell therapy relies critically on the interaction of the donor cells with host tissues to restore function.

The first widely used stem cell therapy in humans was bone marrow transplantation. In the late 1950s, Thomas et al. demonstrated that in two patients with leukemia, infusion of bone marrow from a healthy identical twin after total-body irradiation resulted in full reconstitution of the bone marrow and temporary remission.[23] Since then, bone marrow and hematopoietic stem cell transplant has become the standard treatment after myeloablation. In addition to repopulating the bone marrow after irradiation, allogeneic stem cell transplantation may further improve treatment outcomes through a graft-versus-tumor effect.[24,25]

As the use of stem cell transplants for reconstituting the immune system has increased, research has also expanded toward injection of other cell types into tissues, including solid organs. Clinical trials are currently underway using stem cells for regeneration of bone, cartilage, and cardiac tissue as well as for treating cancer, hematologic diseases, diabetes, and neurodegenerative disorders.[26] The first approved clinical trial using cells derived from ESCs for treatment of thoracic spinal cord injury was initiated in 2010, and additional phase I/II trials are ongoing for treatment of macular defects using retinal pigment epithelium derived from ESCs.[26]

The greatest limitations in stem cell therapy are the low survival rates of the injected cells and the inability to closely control the location of those cells that do survive. Studies examining the effects of the delivery of MSCs after myocardial infarction have shown that the majority of cells injected intravenously were eventually found in the lungs, spleen, and liver, with only a small percentage of the cells engrafted into the injured heart wall.[27–29] It is possible that some of these limitations could be overcome by injecting cells directly into the tissue; however, this increases the risk of further damage, and cell survival is still limited with this method.

Although cell therapies are currently limited by low percentages of cell engraftment, significant functional improvements are often still seen. Therefore, it has become widely accepted that it is not necessarily the differentiation and direct engraftment of the injected cells themselves that result in improved function but rather the autocrine and paracrine effects of the smaller number of cells that do survive and engraft. Therefore, although significant benefit may be derived through increasing survival and localization of stem cells after injection, understanding the mechanisms through which cells do provide benefit via trophic influences may eventually be of substantially greater value.

1.3.2 Tissue Engineering and Biomaterials

In tissue engineering, biomaterials serve as the scaffolding upon which cells build tissues. The definition of biomaterials has recently been revisited and is now interpreted as encompassing natural or synthetic materials that interact with biological systems.[30] The use of biomaterials in medicine has been around for centuries, with dental implants made from wood and contact lenses made from glass being some of the first common applications of biomaterials. Because early implants were designed to remain in place for long periods of time and little was known about the mechanisms behind the foreign body response, initial biocompatibility studies in the

1940s focused on determining which materials were the least chemically reactive. However, this changed with the development of applications in which it was desirable for the biomaterial to interact directly with the host tissue as well as degrade over time. Therefore, the definition of *biocompatibility* has become focused on materials having an "appropriate host response" rather than limiting the response.[31] Today, in addition to being biocompatible, biomaterials in tissue engineering applications have become increasingly sophisticated and are designed to meet several criteria. First, they should provide appropriate mechanical strength to ensure that the tissue can withstand the normal forces it experiences or perform its physical functions *in vivo*. Second, they must provide a compatible surface for cell attachment and appropriate topographic information. Third, they should ideally be designed to degrade over a length of time that is appropriate for the specific application, such that ultimately, the engineered tissue is able to approximate its native state.

Synthetic polymers have an advantage over natural polymers as biomaterials for tissue engineering because they may be produced using defined processes and have highly tunable mechanical and chemical properties to enhance biocompatibility. However, nature's biomaterial—the extracellular matrix (ECM)—already possesses the optimal properties to support cellular attachment and tissue growth, often in a tissue-specific manner. This has led tissue engineers to study in depth the structure and composition of the native ECM as well as investigate cell–material interactions with the goal of recreating this environment.

The native ECM is a complex and dynamic network of proteins that provides both structural and biochemical support to the cells it surrounds.[32] Rather than just serving as a passive scaffold, the ECM also provides important mechanical, topographic, and biochemical cues that can influence cell attachment, survival, shape, proliferation, migration, and differentiation.[33] The most abundant protein in the ECM is collagen, which makes up approximately 30% of the total protein in the human body. Mature collagen is a triple helix of three polypeptides that align and combine themselves to form collagen fibrils that are typically between 50 and 500 nm in diameter.[34,35] Other fibrous proteins such as fibronectin, laminin, and elastin are also present in significant quantities and influence the structural and mechanical properties of the tissue. In addition to the fibrous proteins, the ECM also contains glycoproteins as well as bound or entrapped growth factors that can significantly influence the properties of the tissue. Each component of the ECM influences cell behavior via specific interactions, often involving ligand-specific receptors on the cell membrane. Therefore, recapitulation of the structure of the native microenvironment using biomaterials with nanoscale features may provide the optimal biomimetic topographic structure for cells to form tissues with similar properties to the native tissue.

There are two levels of interactions that must be investigated to develop the optimal tissue engineered solution for clinical use: (1) the cell–material interactions *in vitro* after initial cell seeding and (2) the interactions of the tissue-engineered constructs with the host tissues after they are implanted. Although the ultimate goal of tissue engineering research is to develop a construct that can be implanted and function *in vivo*, it is imperative to first gain a thorough understanding of the cell–

scaffold interactions in well-defined *in vitro* environments. Tissue engineering research currently focuses on applying knowledge of the biological characteristics of native cellular and tissue microenvironment to the development of biomaterial-based constructs that mimic these behaviors when combined with cells.

1.3.3 Bioactive Factors in Tissue Engineering

In addition to the cell source and scaffolds, the use of bioactive factors is important for the optimization of tissue-engineered constructs. Although the ECM provides the structural component of the native tissue, it also contains soluble bioactive factors, such as growth factors and cytokines, whose signals direct aspects of cell behavior, including survival, proliferation, migration, and differentiation. *In vivo*, these biofactors are secreted by cells and exert their effects by binding to the receptors of target cells and stimulating signal transduction to alter gene expression. The effects of growth factors are dependent on the identity and state of the target cells as well as the structure and composition of the ECM. Different cell types can have different responses to the same growth factor, and one or more growth factors may induce the same downstream effects.

The behavior of growth factors is often modulated by the ECM, which can control the activity of growth factors in several ways. The effects of growth factors are dependent on the concentration of their active forms. Binding to components of the ECM, such as proteoglycans, can extend the stability of growth factors by protecting against proteolytic degradation and thus maintain effective concentrations. Alternatively, growth factors may be secreted in an inactive form and require cleavage or co-factor binding in the ECM to become activated.[36]

A large number of *in vitro* studies have successfully used growth factors to direct differentiation of stem cells down specific cell lineages. However, it may be difficult to effectively control differentiation *in vivo.* Specifically, intravenous administration of growth factors may be undesirable and largely ineffective since repeated infusions of high concentrations of growth factors would be required due to the short half-life of the factors, which may lead to negative systemic effects.[37] Therefore, the development of tissue engineering strategies that control the availability and limit the degradation of growth factors have gained increasing importance.

1.4 NANOTECHNOLOGY IN REGENERATIVE MEDICINE AND TISSUE ENGINEERING

1.4.1 Introduction to Nanotechnology

The human body is a complex, dynamic system with tissues and organs regulated at the subcellular level. Although there have been some successes in translating regenerative medicine and tissue engineering techniques to the clinic, they are few and are typically limited to a single or only a handful of patients. Progress in the field has been limited by the difficulty in recreating the complex interactions in

the native tissue environment that are needed to ensure functional tissue regeneration. As more is learned about the mechanisms that control tissue growth and formation, it has become clear that the answer most likely lies in controlling the cell behavior at the nanoscale. Although complete *in vitro* recreation of the *in vivo* environment remains a somewhat distant target, this may not be necessary for success. Instead, it is important that we aim to understand the interactions between cells and their native environment and apply this knowledge to the development of constructs that will jumpstart tissue formation down the correct path before allowing the most effective incubator—the human body—to take over and remodel the engineered cells or constructs into the optimal structure.

Because the individual chapters in this text will provide in-depth discussion of the different applications of nanotechnology in this field, we provide here a brief overview of past and current research that has used nanoscale approaches in tissue engineering and regenerative medicine.

1.4.2 Nano-Based Cell Tracking

Currently, the main application of nanotechnology in cell therapy is in cell tracking. Although histologic methods have traditionally been used to determine cell localization after implantation in animal models, these methods require harvesting, dissection and processing of the tissue, which is undesirable after implantation into humans. Recent developments in imaging technologies using nanoscale labeling methods that allow cells to be imaged *in vivo* and tracked in real time without compromising their function can improve understanding of cell fate after implantation and aid in future studies to improve cell engraftment and survival.

There are two main types of nano-based labeling techniques that have been investigated for noninvasive *in vivo* imaging: magnetic nanoparticles and fluorescent nanoparticles. Magnetic nanoparticles are commonly composed of superparamagnetic iron oxide (SPIO) particles 50–500 nm in diameter, which show enhanced contrast under magnetic resonance imaging (MRI).[38] SPIO particles have been successfully used for cell tracking in stem cell therapy studies in animals[39–41] and humans.[42,43] Although initially it was thought that SPIO particles did not affect cell behavior, recent studies suggest that exposure to magnetic fields after labeling may alter adipogenic and osteogenic differentiation capacity.[44] Further studies examining the effects on cells with SPIO particles under MRI are important before their clinical use.

Traditional fluorescent cell-labeling methods are limited by their lack of photostability, narrow excitation range, cell and tissue autofluorescence, and broad emission spectra.[45] Quantum dots, or Q-dots, are fluorescent nanoparticles with diameters that typically range from 2 to 5 nm and can be synthesized in any color.[46] Q-dots have greater photostability and a narrower emission profile than traditional fluorescent dyes, and they can be linked to specific proteins or DNA sequences to monitor specific cell behaviors.[47] Although *in vivo* studies using Q-dots are currently limited, early studies show that labeled cells can be tracked when injected into

mice.[48,49] However, there is some concern that they may alter cell function and differentiation, which may limit their use in a clinical setting.[46,50]

It is important to note that addition of genetic information to cells via viral particles, also known as gene therapy, is also a common method being tested in stem cell therapy to direct stem cell fate or to label cells for *in vivo* imaging. Viral vectors themselves may be considered a nanobiomaterial because they are typically engineered to deliver specific genes.[51] This important topic will not be explored in this chapter, which focuses instead on tissue engineering and regenerative medicine based on nonviral approaches.

1.4.3 2D Nanotopography

To examine the effects of topography on cells in a controlled environment, a large number of studies have fabricated substrates that contain microscale and nanoscale features on otherwise 2D surfaces. Results suggest that seeding cells onto substrates with micro- or nanoscale ridges, grooves, posts, or pits can affect cell attachment, shape, migration, proliferation, and differentiation.[52] Recently, mathematical algorithms have been used to generate a "chip" with thousands of random topographies and seeded with MSCs to examine the effects on proliferation and differentiation.[53] Application of these screens to determine the effects of varying topography may prove to be extremely valuable to understanding the optimal surfaces for controlling cell growth and differentiation.

In addition to physical topography, nanoscale manipulations of the biochemical structures can also strongly influence cell behavior. When biomaterials are either used in *in vitro* culture or implanted *in vivo*, the material surface becomes rapidly coated or opsonized with proteins before cell attachment. In both the native environment as well as on scaffolds, cells interact with ECM proteins via heterodimeric transmembrane receptors known as integrins. When integrins come into contact with specific peptide sequences on ECM molecules, they cluster and recruit other proteins to form the focal adhesion complex and signal to downstream effectors, which then influence cellular behavior.[54–56]

One of the most common integrin binding sequences is the arginine-glycine-aspartic acid (RGD) sequence. By modifying a substrate with the RGD peptide, cell adhesion, proliferation, and migration can be altered.[57] Because obtaining substantial quantities of purified proteins is difficult and may cause adverse host immune responses, the use of short synthetic peptides that are not recognized by the host's immune system but still contain functional domains to modulate cell adhesion to scaffolds is an attractive method for tissue engineering. However, since the concentration and distribution of cell adhesion peptides are both likely to significantly affect cell behavior, it is important to optimize these parameters for specific applications. For instance, it has been shown that clustering of RGD molecules into groups of nine improved cell motility compared with groups of five or individual molecules, independent of overall RGD concentration.[58]

Although the RGD molecule is a common receptor for many different integrins, integrin specificity for full-length proteins is determined by the configuration of

multiple regions within the protein. This is important because each integrin exerts specific intracellular downstream effects that differentially influence cell behaviors. For example, it has been shown that varying surface chemistry can influence whether cell attachment is mediated through binding to integrin $\alpha_5\beta_1$ or integrin $\alpha_v\beta_3$.[59,60] One study showed that when RGD alone was presented to cells, or with its synergy site PHSRN, adhesion was mediated by $\alpha_V\beta_3$. However, when a longer purified recombinant region of the fibronectin protein was used, osteoblast adhesion occurred through integrin $\alpha_5\beta_1$, suggesting that the tertiary structure of the protein may be important to consider when designing scaffolds for tissue engineering that specifically control cell behavior.[61]

Studies using surfaces modified to have nanoscale features or express specific cell-adhesion peptides provide a highly organized way of examining cell behavior with topographic and biochemical cues and give important insights into the interactions of cells with textured surfaces. However, these substrates are limited in their complexity compared to the native tissue environment. The textured surfaces only provide cues to the portion of the cell that is in contact with the surface and essentially limit cell motility to two dimensions. Although this is relevant for epithelium and vasculature endothelium, for most other tissues, it does not closely mimic the native environment. Additionally, the time course of these studies is typically short, making it impossible to examine key activities in tissue formation, such as ECM deposition and modification. Therefore, tissue engineering studies aimed at building a functional tissue *in vitro* typically focus on developing and using porous scaffolds where cells are surrounded on all sides by the scaffold material, similar to the native ECM, and the constructs are maintained in culture for several weeks to allow time for protein deposition and remodeling.

1.4.4 3D Nanoscaffolds

Extensive studies have shown that there are clear differences in cell behavior and tissue formation on flat surfaces compared with 3D ECM scaffolds and that these differences occur at the protein and subprotein level.[62–65] Furthermore, these differences are present even when the 2D scaffold has the same chemical and molecular composition.[63] These findings, in combination with the evidence that nanoscale topography affects cell behavior, have pushed scientists to use tissue-engineering approaches that mimic the 3D submicron structure of the native ECM. The currently available techniques for scaffold fabrication do not approach the complexity of the native ECM structure, nor can the environment be as closely controlled as with the 2D nanotopography described earlier. However, this may not be necessary because scaffolds in tissue engineering studies are designed to act primarily as a temporary structure to support cells until they are able to synthesize their own functional ECM. Therefore, the goal in successful application of 3D tissue engineered scaffolds is to identify and enhance key components that will provide the appropriate signals to cells to generate a functional tissue that can be translated into clinical use.

Nanobiomaterial constructs can be made using a wide variety of materials and methods to create scaffolds with many different structural and chemical properties that can be tailored to the specific application. As described earlier, the native ECM is composed predominantly of nanofibrous proteins. Therefore, efforts to recreate an environment similar to the original tissue have heavily focused on the development of fibrous scaffolds. A brief overview of the most common methods of synthesizing nanofibrous scaffolds is provided next. Other chapters in this volume present more in depth descriptions of these techniques and their applications in tissue engineering.

1.4.4.1 Phase Separation Thermally induced phase separation (TIPS) is a technique that is particularly useful for generating scaffolds with a specific pore size. In this method, the temperature of the polymer solution is adjusted to a point at which a "polymer-rich" and a "polymer-poor" phase is generated. The solvent is removed, and the polymer-rich phase solidifies, forming a porous solid structure, which is then freeze dried. Nanofibrous scaffolds with varying fiber diameters and pore sizes can be generated by adjusting the polymer concentration, the type of solvent, and the phase separation temperature.[66] Specifically, fibers ranging from 50 to 500 nm in diameter—similar to the size of native collagen—can be produced. Additionally, highly controlled interconnected macroporosity can be introduced by pouring the polymer solution over a negative wax mold, which is removed after solidification of the polymer.[67]

Nanofibrous scaffolds generated with TIPS have been shown to display increased attachment of seeded osteoblasts compared to solid wall scaffolds with similar macroporous architecture.[67] Additionally, mouse ESCs seeded in TIPS nanofibrous scaffolds and cultured under osteogenic conditions expressed higher levels of osteogenic genes and showed immunohistochemically detectable greater immunofluorescence than cells cultured on flat surfaces.[68]

TIPS is a simple and reproducible method for scaffold synthesis that does not require any specialized equipment. Additionally, it can be used to form complex 3D shapes that can be designed for an individual patient (Fig. 1.3).[67] However, it also has some important limitations. First, the technique can only be used with a limited number of polymers, and second, it would be difficult to scale up to a commercial level.[69]

1.4.4.2 Self-Assembly Another method of generating nanofibrous scaffolds that mimic the structure of the ECM is through self-assembly of peptide amphiphiles (PAs). This is a bottom-up approach that mimics natural processes such as nucleic acid and protein synthesis. PAs are short peptide structures that spontaneously aggregate into cylindrical micelles approximately 5–8 nm in diameter and 1 μm in length. This process occurs through noncovalent bonds under specifically tailored conditions. The peptides are typically composed of a hydrophobic alkyl chain tail, which forms the inside core of the fiber, and a hydrophilic head composed of epitopes, such as RGD, typically found in the native ECM that face outward, and interact with cells or other components of the ECM (Fig. 1.4). Other structural domains positioned between the tail and head regions of the PA function to stabilize

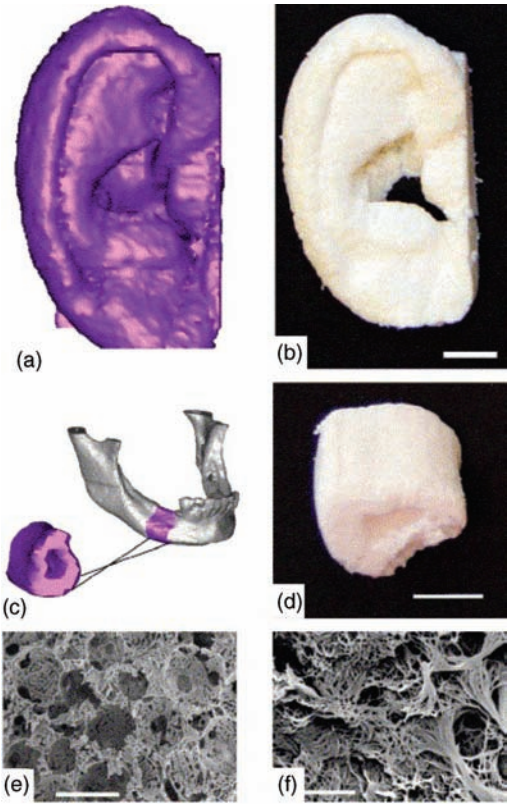

FIGURE 1.3 Scaffolds created using thermally induced phase separation derived from three-dimensional reconstructions of computed tomography (CT) scans. (a) Human ear reconstructed from histological sections and (b) the resulting nanofiber scaffold (scale bar = 10 mm). (c) Human mandible reconstruction from CT scans and (d) resulting nanofiber scaffold (scale bar = 10 mm). (e) Scanning electron micrographs showing interconnected spherical pores within mandible segment (scale bar = 500 μm) and (f) nanofiber pore morphology within a single pore (scale bar = 5 μm). Reprinted with permission from Ref. [67].

the network through hydrogen bonds and include regions that contain charged amino acids that control the solubility of the PAs under different pH conditions.[70] These structural features are particularly useful for generating injectables that are prepared under conditions that prevent self-assembly but are induced to undergo rapid self-assembly into a nanofiber network upon exposure to physiological pH. Bioactive factors such as DNA, drugs, or other proteins may be mixed into the unassembled solution for encapsulation into the fibers upon assembly and then released into the surrounding environment upon degradation.

Self-assembly of PAs can be used to generate a large variety of nanostructures with specifically tuned biochemical and degradation properties. For example, to promote mineral deposition for bone formation, phosphoserine residues have been

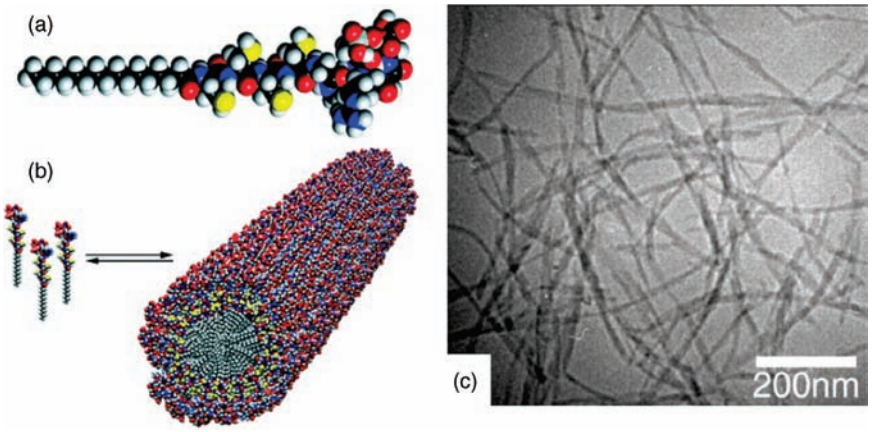

FIGURE 1.4 General structure of self-assembled peptide amphiphile (PA). (a) Molecular model of the PA showing the overall shape of the molecule. The *narrow gray area* represents the hydrophobic alkyl tail and the thicker head region is composed of hydrophilic amino acids containing functional groups that can provide signals to the cells to influence their behavior. (b) The PAs self-assemble into nanofibrous structures upon exposure to physiological conditions with the hydrophobic tail in the core and the head region facing the outside to interact with cells. (c) Vitreous ice cryotransmission electron microscopy image of hydrated PA fibers (scale bar = 200 nm). Modified with permission from Ref. [70].

incorporated into PAs with adhesion-promoting RGD head sequences. These phosphoserine residues serve as a template for nucleation of hydroxyapatite crystals that align along the long axis of the nanofibers, similar to the native bone structure.[71] The RGD peptides on the outer region of the PA promote cell adhesion. Other studies have shown that the use of self-assembled PAs can promote neural regeneration[72] as well as angiogenesis.[73] This technique not only provides biological cues to induce tissue formation but also mimics the basic steps of ECM biosynthesis. However, this method of nanofiber synthesis is quite complex and of relatively low yield and may not be suitable for large-scale tissue engineering applications.[74]

1.4.4.3 Electrospinning Electrospinning has recently become the most commonly used method for the fabrication of nanofibrous biomaterials. This method involves the application of a high electric field to a polymer solution delivered at a constant rate through a needle. At a high enough voltage, the charge on the polymer overcomes the surface tension of the solution and causes emission of a fine polymer jet. This jet undergoes a whipping process, and the fibers are further elongated as the solvent evaporates and fibers are deposited on a grounded collector (Fig. 1.5). Both natural and synthetic polymer scaffolds have been successfully created using the electrospinning method. The ability to generate three-dimensional scaffolds with tailored architecture, mechanical properties, and degradation characteristics has made electrospinning a popular method in tissue engineering applications. Altering parameters during the electrospinning process, such as polymer concentration, flow

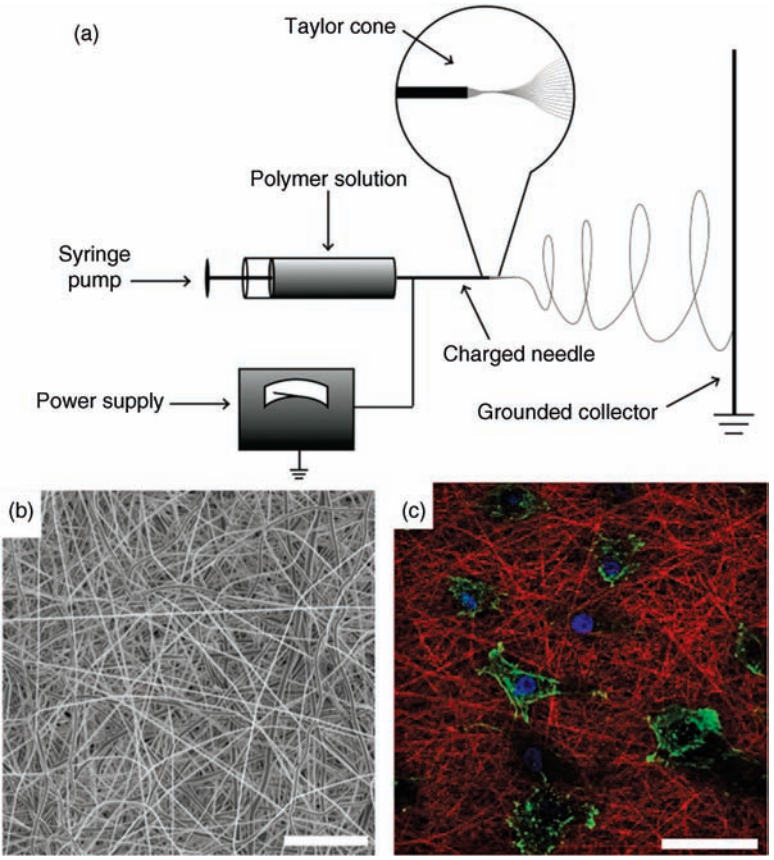

FIGURE 1.5 Fabrication of electrospun nanofibers. (a) General electrospinning setup consisting of syringe filled with polymer solution that is pumped through a needle charged with a high-voltage power supply. When the electrostatic forces between the collector and the solution overcome the surface tension of the solution, the solution is pulled out of the Taylor cone into fine fibers that are deposited on the grounded collector. (b) Scanning electron microscopic image of randomly arranged poly(ε-caprolactone) (PCL) nanofibers formed by electrospinning (scale bar = 20 μm). (c) Fluorescent image of mesenchymal stem cells seeded on nanofibers. Green, cells (membrane label); red, nanofibers; blue, cell nuclei (DAPI stain); scale bar = 20 μm.

rate of the solution, and voltage applied, can generate fibers ranging from approximately 100 nm to several micrometers in diameter. Whereas scaffolds with aligned fibers can be created by collecting fibers on a drum or mandrel rotating at high speeds, randomly oriented fibers are generated on slowly rotating or stationary collectors.

Various polymers have been used in electrospinning of nanofiber scaffolds. These include synthetic polymers such as poly(L-lactic acid) (PLLA),[75–78] poly (ε-caprolactone) (PCL),[79–85] and polyurethane,[86] as well as natural polymers such as

collagen,[87–90] elastin,[87,91] silk fibroin,[92–94] dextran,[95] and chitosan.[96,97] Because the synthetic polymers typically used in electrospinning are hydrophobic and lack biologically active functional groups, they are often modified either physically or chemically after the electrospinning process to increase their hydrophilicity and ability to interact with cells and biomolecules.

Plasma treatment, similar to that performed on tissue-culture polystyrene, can generate functional carboxyl or amine groups on the surface of the fibers. This has been shown to enhance cell attachment and proliferation either alone[98,99] or via the coating of the functionalized fiber with a natural ECM protein such as collagen[100,101] or gelatin.[102] Wet chemical etching methods may provide more homogeneous functionalization in thicker scaffolds because plasma etching can only penetrate the outer surface of a thicker scaffold. This method typically involves NaOH hydrolysis or aminolysis of the polymer, breaking the ester bond at random points and creating a hydroxyl or amino group, respectively.[103] One study demonstrated that esophageal epithelial cells seeded on aminolyzed poly(L-lactide-*co*-caprolactone)(PLCL) coated with fibronectin exhibited higher collagen type IV synthesis than those seeded on the unmodified polymer, suggesting that this method may be useful in tissue engineering studies.[104]

Composite scaffolds formed from co-electrospinning of different polymers have been used to control the mechanical as well as structural properties of the scaffold. Perhaps the biggest challenge using the electrospinning method is that the pore size is typically much smaller than the diameter of a typical cell, a property that makes cell and nutrient infiltration into the middle of the scaffold difficult. Several methods have been used to overcome this problem, including spinning of mixed microfiber and nanofibers scaffolds,[105] as well as using water-soluble polymers (i.e., polyethylene oxide [PEO]) in combination with slower-degrading materials (i.e., PCL), that can be quickly dissolved after spinning, leaving the nonsoluble, slower-degrading polymer behind with larger pore sizes.[106–108]

To more closely tailor the properties of a scaffold—including biologic, mechanical, and degradation characteristics—researchers have begun to combine two or more different components within a single scaffold. This can be done before electrospinning by mixing several polymers within a single solution, which results in a single fiber containing each component or by electrospinning multiple solutions of polymers onto the same collector, thereby creating a scaffold with multiple fiber types.

Although natural polymer scaffolds composed of ECM proteins such as collagen and elastin show increased cellular response, when used alone, they lack sufficient mechanical properties to function in the *in vivo* setting. Combining ECM derived from urinary bladder matrix with poly(ester-urethane)urea, Stankus et al. were able to develop electrospun scaffolds with improved mechanical and biological properties than possible using the individual polymers alone.[109] Similarly, Lee et al. mixed collagen and elastin with several biodegradable synthetic polymers to develop scaffolds to use as vascular grafts.[110]

Some polymers cannot be dissolved in the same solvent, therefore limiting the options for combining several different polymers within the same solution.

Additionally, it may be desirable to have fibers of different dimensions or mechanical properties within the same scaffold. This has led to the development of multi-jet electrospinning in which different polymer solutions can either be electrospun at the same time to generate a homogeneous mixed scaffold or sequentially to generate a layered scaffold.[111] Baker et al. co-electrospun three different solutions containing polymers with varying degradation rates and mechanical properties to develop a scaffold that allowed for both improved cellular infiltration by increasing pore size as well as more closely mimicked the properties of the native tissue.[112]

Electrospinning is a relatively simple, cost-effective technique that has shown significant potential in studies aimed at repair of many different types of tissues. When seeded with stem cells, nanofiber scaffolds have been shown to enhance differentiation toward many different cell types, including bone, cartilage, cardiac and skeletal muscle, blood vessels, and nerve.[113,114]

1.4.5 Growth Factor Delivery

Although the scaffold structure plays an essential role in controlling cell behavior, chemical or biological modulators of cell activity and phenotype heavily influence tissue formation both *in vitro* and *in vivo*. In native tissues, growth factors provide specific signals to cells that direct cell activities, including cell migration, proliferation, and differentiation. The effects of growth factors are quite complex and are dependent on the concentration of the growth factor, phenotype of the cells acted on by the growth factor, and functional characteristics of the specific cell receptor interacting with the growth factor.

In vitro tissue engineering studies often supply relevant growth factors in the culture medium to induce cellular differentiation. However, because most growth factors have very short half-lives, in order to maintain long-term signaling when tissue engineered constructs are implanted *in vivo*, it is important to develop a delivery system that can provide sufficient concentrations of specific factors over the desired period of time, preferably at specified rates. Nanoscale techniques for growth factor delivery have typically focused on two basic methods: (1) immobilization of the growth factor on the surface of a substrate or (2) encapsulation of the growth factor within a degradable delivery system.

Growth factors can be immobilized onto a material surface through either physical adsorption or through covalent linkage. Although simple physical adsorption is limited in its effectiveness because of competition by other proteins with higher affinity for the polymer,[115] successful noncovalent adsorption onto a nanomaterial has been accomplished by mixing heparin into a synthetic polymer solution that is then electrospun into nanofibers. Heparin is a sulfated glycosaminoglycan that has a strong affinity for a number of growth factors, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β). In one study, low-molecular-weight heparin was conjugated to a poly(ethylene glycol) (PEG) carrier and electrospun with either PEO or poly(lactide-*co*-glycolide) followed by successful

adsorption of bFGF.[116] Another study demonstrated that bFGF and EGF adsorbed onto PLLA nanofibers coated with covalently linked heparin maintained their activity and induced neural differentiation and axon growth in human ESCs, but simple adsorption of the growth factors did not have an effect on the cells.[117]

Although noncovalent adsorption is useful if presence of the growth factor is only needed initially, covalent linkage of growth factors to nanofibers is typically preferred for tissue engineering applications because of the slower release profile, which is dependent on the rate of degradation of the polymer to which it is attached. In one study, amine-terminated PCL–PEG block copolymers were electrospun and EGF was covalently immobilized on the PEG-linked amine. In a mouse diabetic wound model, EGF conjugated nanofibers showed enhanced healing compared with unconjugated EGF and nanofibers alone, as well as upregulated EGF receptor expression on the cells in the wound area.[118]

To regulate the release of growth factors from a surface, some investigators have examined the effects of encapsulating the growth factors in nanoparticles, which are then adsorbed onto a nanofiber surface. In one study, PLGA nanoparticles containing platelet-derived growth factor (PDGF) were immobilized onto nanofibrous PLLA scaffolds.[119] Growth factor release from nanoparticles was prolonged when they were immobilized to scaffolds compared with free nanoparticles, and bioactivity was retained over a 14-day period, suggesting that this method may be useful for delivery of growth factors to influence cellular activity in a tissue engineered construct. Additionally, the amount and rate of release of growth factors can be controlled by altering one or more parameters, including the biodegradability of the nanoparticles, the molecular weight of the polymers used, the ratio of lactic to glycolic acid, or the amount of growth factor encapsulated within the nanoparticle.

Incorporation of growth factors directly into the fibers of scaffolds has also shown promise as a method for sustained delivery, although this approach can alter the degradation and mechanical properties of the scaffold and must be considered during synthesis.[120] Another challenge in this strategy is that exposing growth factors to the organic solvents used to generate polymer solutions can denature the growth factors.[121] This is typically solved by incorporation of a hydrophilic additive, such as PEO or bovine serum albumin (BSA), which minimizes the contact between the protein and the organic solvent. Studies have demonstrated that electrospinning solutions containing nerve growth factor (NGF) with BSA and either PCL or PLCL can produce nanofibers that release active NGF over several weeks and promote neurite outgrowth when seeded with PC-12 cells.[122,123] Bone morphogenetic protein-2 (BMP2) was incorporated into silk scaffolds with PEO and demonstrated increased osteogenic differentiation of MSCs and calcium deposition compared with scaffolds without BMP2.[124] A novel method of nanoparticle synthesis using sugar molecules to protect proteins from degradation under harsh environments as well as allow for sustained release of the protein of interest has recently been developed. These nanoparticles have excellent storage stability and can be used with almost any protein or polymer of interest, making them particularly attractive as a method for delivering bioactive factors within a scaffold.[125]

Coaxial electrospinning has also been investigated as a method of growth factor delivery. In this method, two solutions are pumped through concentric needles to form fibers containing an outer shell and inner core of different components. Placing the solution containing the growth factor on the inside of the fiber reduces the potential for denaturation by the organic solvent used to dissolve the outer polymer. One study compared the release of bFGF from electrospun fibers that were prepared by direct blending the bFGF into the polymer solution and by coaxial electrospinning with bFGF in the core of the fiber.[126] Both methods resulted in increased attachment, proliferation, and differentiation of seeded bone marrow stem cells compared with cells on fibers without bFGF. However, coaxial electrospinning resulted in a slower release profile of bFGF compared with the blended method. Another study showed that the protein release rate from a coaxial electrospun fiber could be increased by increasing the feed rate of the core solution or by adding a polymer with a faster rate of degradation (i.e., PEG) to the outer shell solution.[127] The ability to tailor both the mechanical and degradation properties of the scaffold as well as control the release rate of growth factors from the scaffold makes this an attractive method for use in future tissue engineering studies.

1.5 CONCLUSIONS

The ultimate goal in regenerative medicine and tissue engineering is to develop technologies to repair or replace tissues without the complication of chronic immunosuppression and dependence on organ donors. The key to success is understanding of how native tissues function and applying this information to establish the proper combination of cellular, structural, and chemical components that will allow for functional tissue development. Although perfect mimicry of the complex tissue structure found in nature is unlikely to be reached soon, it is critically important to gain a fuller understanding of how cells receive the signals needed to achieve the appropriate phenotype and to form functional tissues once implanted *in vivo*. It is increasingly apparent that such investigations will need to transcend the tissue, or even the cellular level, and take into consideration nanoscale phenomena that control interactions between cells, scaffolds and bioactive substances. Significant advances have been made both in deciphering the biology behind cell–matrix interactions as well as generating artificial ECM and controlling stem cell fate in the laboratory; however, significant improvements remain necessary to make regeneration of tissues a widespread clinical option. This chapter provides an abbreviated overview of the exciting developments and conceptual and practical challenges in cell-nanomaterial biology and engineering that is explored in depth in the chapters of this book.

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2

NANOFIBER TECHNOLOGY FOR CONTROLLING STEM CELL FUNCTIONS AND TISSUE ENGINEERING

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2.1 INTRODUCTION

Nanotechnology is an upcoming yet promising technology with respect to the development of well-established products. It holds the potential to create new products with novel characteristics and functions in a wide range of applications.

Micro and Nanotechnologies in Engineering Stem Cells and Tissues, First Edition. Edited by Murugan Ramalingam, Esmaiel Jabbari, Seeram Ramakrishna, and Ali Khademhosseini.

Application of nanotechnology in life sciences research, particularly at the cellular level, sets the stage for an exciting role of nanotechnology in nanomedicine for health care. The potential medical applications are predominantly in detection, diagnostics (disease diagnosis and imaging), monitoring, and therapeutics. The availability of more durable and better prosthetics and new drug-delivery systems are of great scientific interest and give hope for cancer treatment and minimum invasive treatments for heart disease, diabetes, and other diseases.[1] Nanofibers are potentially recent additions to materials in relation to tissue engineering (TE). Tissue engineering is the application of knowledge and expertise from a multidisciplinary field to develop and manufacture therapeutic products that use the combination of matrix scaffolds with viable human cell systems or cell responsive biomolecules derived from such cells for the repair, restoration, or regeneration of cells or tissue damaged by injury, disease, or congenital defects.[2]

Tissues in the body are made up of cells and insoluble materials present between the cells known as the extracellular matrix (ECM). ECM is composed of various biomacromolecules secreted by surrounding cells and is responsible for the structural support and tensile strength of the tissue. It provides a substrate for cell adhesion and migration and regulates cellular differentiation. The interaction between cells and ECM is mediated by the process of biorecognition whereby the transmembrane protein receptors on the cell membrane combine specifically with specific ligands in the ECM, triggering a series of events in the signal transduction cascade within the cells and eventually influencing their gene expression. For example, growth factors such as fibroblast growth factor combine with their receptors on cell surfaces and stimulate their proliferation and differentiation.[3]

Recently, nanofiber-based scaffolds are being explored as scaffolds for tissue engineering applications. TE is an interdisciplinary field of research whereby diverse cell-based and cell-free strategies are being investigated in the quest for a sustainable therapeutic for refurbishment of organ functionality. Essentially, TE is an attempt at bringing about repair by mimicking nature. It is aimed at boosting the low regenerative capacity of the damaged myocardium by applying principles of engineering, material chemistry, and cell biology. The classical strategy used in tissue engineering is the provision of external help in the form of biomaterials and biomolecules with properties bearing close resemblance to their natural counterparts. However, owing to the uniqueness of each organ, the quest for optimal biomaterials and an efficient strategy for TE remain persistent. A bioengineered construct is desired to possess certain essential characteristics, such as appropriate physical and mechanical properties, ready adherence, nontoxicity, nonantigenicity, noninvasive applicability, and ability for complete integration with the host.[4,5] An ideal polymeric scaffold satisfies several structural and chemical features: (1) a three-dimensional architecture with a desired volume, shape, and mechanical strength;[6] (2) a highly porous and well-interconnected open pore structure to allow high cell seeding density and tissue ingrowth; (3) chemical compositions such that its surface and degradation products are biocompatible, causing minimal immune or inflammatory responses;[7] and (4) their degradation rate finely tuned in a pattern that it provides sufficient support until the full regrowth of impaired tissues. Several scaffold fabrication techniques, namely, electrospinning (random, aligned, vertical, and

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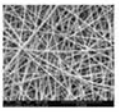
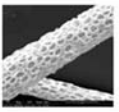
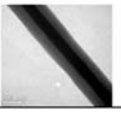
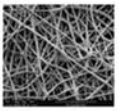
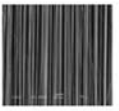
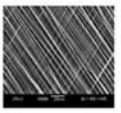
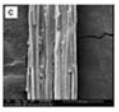
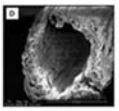
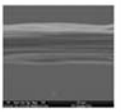
Morphology	Solid 	Porous 	Core-shell 
Assembly	Random 	Aligned 	Layered 
	Yarn 	Hollow yarn 	Fiber bundle 

FIGURE 2.1 Different types of electrospun fibers. PCL = poly(ε-caprolactone); PHBV = poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate); PLGA = poly(lactic-*co*-glycolic acid); PLLA = poly(L-lactic acid).

core shell nanofibers), self-assembly, phase separation, melt-blown, and template synthesis, are used for the preparation of nanofibrous materials (Fig. 2.1). This makes designing of nanofibrous scaffolds an important technique for designing synthetic and natural nanofibers in tissue engineering. It is highly advantageous to have an artificial ECM that promotes cell adhesion and that can be assimilated by the body as the new tissue regenerates. For regeneration of tissues, cell adhesion has been proven beneficial and can be achieved by suitable modifications of biomaterial surface chemistry such as addition of arginine–glycine–aspartic acid (RGD) moieties or growth factors for cell attachment or chemotactic recruitment. Attempting to fabricate artificial ECM, each approach has its own unique characteristics and has the potential to accommodate cells and guide their growth for tissue regeneration.[8] However, electrospinning is the most widely accepted technique; it seems to be demonstrating most promising results for tissue engineering applications.[9] Nanotechnology is also extended as drug-delivery and drug-targeting systems. Owing to the smallness of nanomaterials, they have the ability to be delivered into the human body with ease. They migrate through cell membranes beneath a critical size and are able to pass and develop nanoscaled ferries, which transport high potential pharmaceutics precisely to their destination.[10] Nanostructured biodegradable polymeric materials act as alternative candidates used to promote a new concept of chemotherapy that may include sustained chemotherapy, controlled and targeted chemotherapy, personalized chemotherapy, and chemotherapy across the various physiological drug barriers such as the gastrointestinal barrier for oral chemotherapy

and the blood–brain barrier for brain tumors.[11] Currently, there is a huge demand for controlled-release polymer systems, and the worldwide annual market exceeds $60 billion. Electrospinning has developed into a versatile technique to fabricate polymeric nanofiber matrices, and the ability to incorporate bioactive therapeutic molecules without adversely affecting their structural integrity and biological activity using the mild electrospinning process has generated significant interest in polymeric nanofiber-based drug release patterns by changing the mode of encapsulation as well as by varying the matrix polymer.[12]

2.2 FABRICATION OF NANOFIBROUS SCAFFOLDS BY ELECTROSPINNING

Electrospinning generates a nonwoven mat of polymeric nanofibers from an electrostatically driven jet of polymer solution. The basic elements of an electrospinning system involve (1) a high-voltage supplier (2–40 kV), (2) a source electrode and grounded collector electrode, and (3) a capillary tube with a needle of small diameter. Electrospinning may be carried out with polymer solution as well as polymer melt for fabrication of nanofibers. The morphology and fiber diameter of the electrospun nanofibers can be controlled by varying the parameters, such as applied electric field strength; spinneret diameter; distance between the spinneret and the collecting substrate; temperature; feeding rate; humidity; air speed; and properties of the solution or melt, including the type of polymer, and polymer molecular weight, such as surface tension, conductivity, and viscosity, depending not only on the temperature but also on the concentration of the sample.[13] The advantage of an electrospun nanofibrous scaffold includes an extremely high (favorable) surface-to-volume ratio, appropriate porosity, and malleability to conform to a wide variety of sizes, textures, and shapes of superior architecture[14] (Fig. 2.2).

In addition, scaffold composition and fabrication can be controlled to confirm desired properties and biofunctionalities. The design and development of nanofibrous scaffolds for tissue engineering approaches involve the understanding of biological processes that are mainly aimed at a conducive to ECM. Many studies were also focused on the understanding and evaluations of several cell-scaffold interactions.[15] Interaction between the stem cells and nanofibers are crucial in a cell-scaffold matrix while using them for different tissue engineering applications. Because the nanofibrous scaffolds are highly porous and the pore size is smaller than the normal cell size, scaffolds might inhibit cell migration. Despite this, studies showed the capability of nanofibrous meshes to infiltrate cells. Cells entering into the matrix through amoeboid movement to migrate through the pores can push the surrounding fibers aside to expand the pore. Scaffolds constructed from naturally occurring proteins, such as collagen, allows much better infiltration of cells into the scaffold than the synthetic polymeric nanofibrous scaffolds.[16] The low-molecular-weight peptides (tripeptide and tetrapeptide) found in ECM proteins, such as laminin, fibronectin, collagen, and vitronectin, are found to modulate the cell behavior to a higher extent. Immobilizations of these

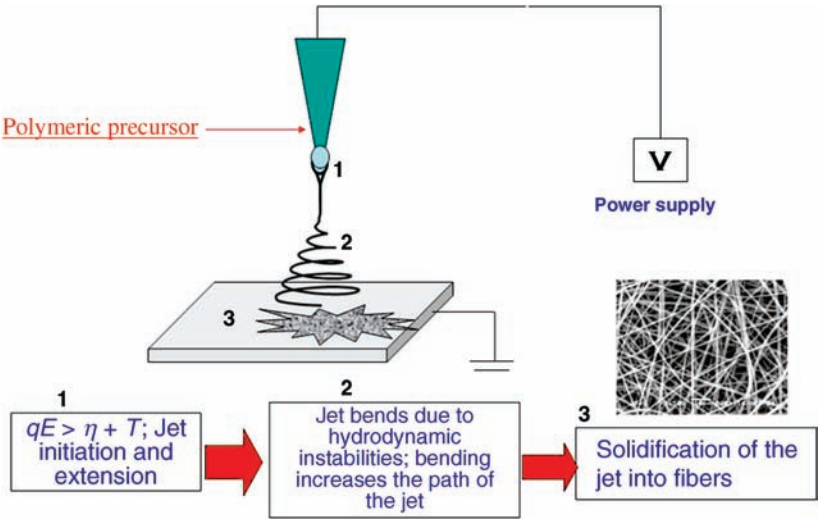


FIGURE 2.2 Schematics of the electrospinning process. The experimental setup consists of a high-voltage power supply, a spinneret, and a collector. The three processes—formation of tailor cone (1), bending caused by various instabilities (2), and collection of solid samples (3)—are shown. The qE is the electrostatic force, η is the viscosity, and T is the surface tension. Conventionally, electrospinning produces a fiber cloth consisting of randomly oriented nano- or microfibers, a typical SEM image of which is also shown.

biological motifs on synthetic biomaterial surfaces are also studied by few researchers so as to increase the bioactivity of the scaffolds. Moreover, the dynamic architecture of the fibers allows cells to adjust according to the pore size and grow into the nanofiber matrices.[17] For many tissue engineering applications, nanofiber modifications are therefore necessary to achieve the required scaffold properties. Polymer blending, coelectrospinning, multilayering and mixing for nanofiber production or cross-linking, surface modifications, and coating of the scaffold can improve the stability and biocompatibility of the scaffold. Multilayering electrospinning is a subset of the electrospinning process that involves sequential electrospinning of polymers on the same collector. This produces multilayered meshes with hierarchically ordered layers made from particular fibers. For example, a trilayered electrospun mesh composed of type I collagen, styrenated gelatin, and segmented polyurethane was prepared; a bilayered tubular construct composed of a thick segmented polyurethane microfiber mesh as an outer layer and a thin type I collagen nanofiber mesh as an inner layer was also fabricated.[18] Alternatively, in a multicomponent mixing electrospinning process, different polymers are simultaneously electrospun from different syringes under special conditions. The produced fibers are mixed on the same collector, resulting in the formation of a mixed fiber mesh (e.g., mixed electrospun fiber mesh composed of segmented polyurethane and polyethylene oxide). Specific

cells are sensitive to pore sizes, and hence high importance is given to polymeric scaffolds with greater porosity. Such porous membranes may be created by phase separation methods.[19] The phase separation method is based on the thermodynamic demixing of a homogenous solution of polymer in solvent into polymer-rich and polymer-poor phases by exposure to another immiscible solvent or by cooling the solution below the glass transition temperature (T_g) of the polymer. Fabrication of scaffolds is influenced by processing variables such as the polymer type, concentration, solvent, and temperature. This method allows the generation of three-dimensional (3D) porous networks within the scaffolds with higher control over porosity and morphology.[20,21]

Physical patterning techniques such as reactive-ion etching and polymer molding allow creation of microgrooves for designated cellular orientations. Patterned surfaces are advantageous as TE scaffolds because they serve better cellular attachment, migration, and orientation.[22] Soft lithographic techniques have been used to generate exquisite control over protein and cells in spatially defined patterns. Such patterning has been shown to regulate the temporal and spatial distribution of biomolecules and has been performed to direct explicit cell behavior and functions. Patterning is also carried out using methods such as imprint lithography, photo or electron beam lithography, and microcontact printing. These methods aid in constructing geometrically designed substrates suitable for cellular interaction on a nanoscale. Imprint lithography method uses a silicone rubber stamp inked with molecules to transfer the agent and develop grids, honeycomb networks, dots, and patterns.[23] These patterns mimic the basement membrane structures of nanometer-sized pores that define mechanical cues that aid specific cell type. However, nanoimprint lithography is capable of creating patterns of submicron 10 nm features with simpler equipment and convenient processing steps.[24] The TE scaffolds designs may be tailored to have specific nanotopographical patterns based on the specific tissue needs by application of the various methods available.

2.3 STEM CELLS: TYPE, ORIGIN, AND FUNCTIONALITY

Stem cells are cells of mammalian origin that possess two specific distinct characteristics: self-renewal and the potential to differentiate into several cell type. In a developing embryo, stem cells can differentiate into all the specialized cells but also maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues. In an adult, stem cells act as a repair and replenish system for all cell types. Stem cells are broadly classified as ESCs, obtained from inner cell mass of an embryo and adult stem cells (ASCs), that are obtained from adult tissues (Fig. 2.3). Stem cells can be cultured *in vitro* and transformed into specialized cell types with characteristics consistent with cells of various tissues such as bone, cartilage, muscle, or nerve after being acquired from an embryo or adult. Recent research demonstrates the development of ESCs such as cells from adult somatic cells by transfusion of pluripotent genes. These are called induced pluripotent stem (iPS) cells.

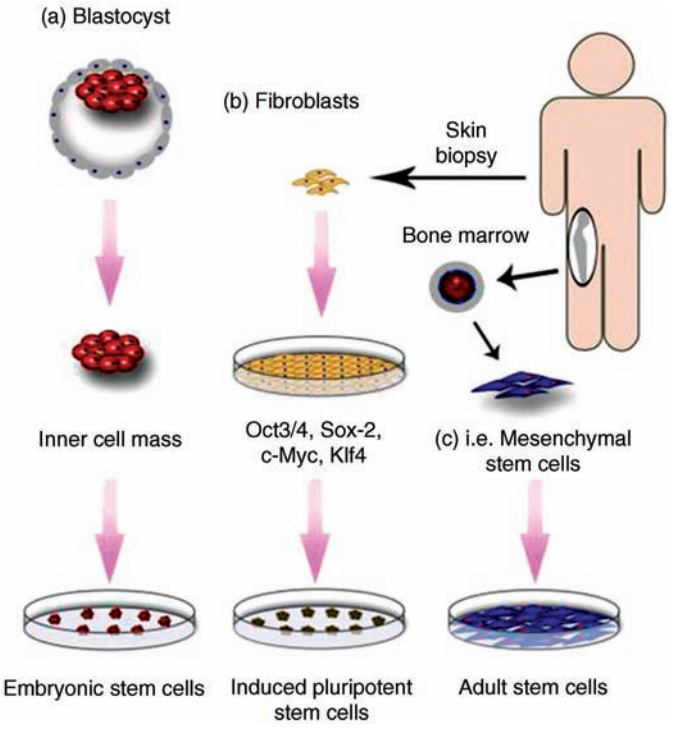


FIGURE 2.3 Stem cell biogenesis. (a) Embryonic stem cells, derived from the inner mass of the blastocysts, are pluripotent cells that may differentiate toward all cell types. (b) Induced pluripotent stem cells generated *in vitro* from somatic cells overexpressing Oct3/4, Sox2, c-Myc, and Klf4. (c) ASCs are created during ontogeny (e.g., bone marrow mesenchymal stem cells) and persist within the niche in most adult animal tissues and organs. Reproduced with permission from Ref. [44].

2.3.1 Mesenchymal Stem Cells

The term mesenchymal stem cells (MSCs) was used by Caplan in 1991 to describe the adherent cells derived from bone marrow that were capable of self-proliferation as well as differentiation into different lineages of connective tissue.[25] However, the identification of these cells dates back to 1867, when Cohnheim identified these cells as nonhematopoietic cells. Traditionally, MSCs were thought to reside in both blood and bone marrow.[26] However, recent researches provide evidence of MSCs in diverse tissue and organs such as lung, adipose tissues, amniotic fluid, umbilical cord, periosteum, dental pulp, hair follicle, thymus, and trabecular bone.[27,28] MSCs give rise to connective tissues of various origin such as bone (osteogenic), cartilage (chondrogenic), and fat (adipogenic).[29] MSCs are also worthy of giving rise to several other tissues of mesodermal (myocyte, osteocyte, endothelium, cardiomyocyte), ectodermal (neuronal), and endodermal (hepatic, pancreatic, respiratory epithelium) lineages.[30] MSCs constitute approximately 2–3% of the total nuclear

cell fraction of the bone marrow. Bone marrow–derived MSCs pose advantage in regenerative medicine because they are naturally poised to generate a particular tissue, which might consist of several cell types such as adipocytes, chondrocytes, osteoblasts, tenocytes, myoblasts, and neurocytes.[31] MSCs express CD44, CD73, CD90, and CD105 receptors while lacking hematopoietic stem cell markers such as CD34 and CD45. MSCs exhibit low expression of major histocompatibility complex (MHC) class I and are negative for MHC class II antigens.[32] Various studies have shown that *in vitro* expanded MSC preferentially home to sites of tissue damage, where they enhance wound healing, support tissue regeneration, and restore the bone marrow microenvironment.[33] However, the exact signaling events that drive MSCs toward this repair mechanism are unknown. MSCs have been applied as therapeutic agents for tissue repair owing to their immunomodulatory properties.[34] All of these properties of MSCs make them an ideal cell source for tissue engineering.[35]

2.3.2 Embryonic Stem Cells

ESCs are isolated from the inner mass of blastocyst cells.[36] Under defined conditions, ESCs are capable of propagating themselves indefinitely. This allows ESCs to be employed as useful tools for both research and regenerative medicine, because they can produce limitless numbers of themselves for continued research or clinical use.[37] Human ESCs are known to express antigens such as octamer binding protein (Oct-4), Nanog, alkaline phosphatase, LIN28, rex-1, crypto/TDGF1, SOX2, and stage-specific embryonic antigen (SSEA) 3 and 4. They also show high levels of telomerase activities.[38,39] It is understood that Oct-3/4 along with SOX2 and Nanog play a crucial role in the process of self-renewal,[40] whereas genes such as Klf4 and c-Myc are involved with maintenance of pluripotency.[41] Because of their plasticity and potentially unlimited capacity for self-renewal, ESC therapies have been proposed for regenerative medicine and tissue replacement after injury or disease. Diseases that could potentially be treated by pluripotent stem cells include a number of blood- and immune system-related genetic diseases, cancers, and disorders; juvenile diabetes; Parkinson's disease; blindness; and spinal cord injuries. Besides the ethical concerns of stem cell therapy, ESCs face certain major technical challenges such as histocompatibility and graft-versus-host disease.

2.3.3 Induced Pluripotent Stem Cells

A few years ago, a completely new class of stem cells was introduced by Takahashi and Yamanaka.[42] The group demonstrated that uptake of genes such as Oct-3/4, Sox2, c-Myc, and Klf4 induces pluripotent properties in somatic cells. These reprogrammed cells were termed iPS cells.[42] Currently, many researchers are actively studying the generation of iPS cells from various sources and trying to improve the experimental procedures.[43] iPS cells are similar to natural pluripotent stem cells, such as ESCs, in many respects, including the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, and potency and

differentiability, but the full extent of their relation to natural pluripotent stem cells is still being assessed.[44] Although additional research is needed, iPSCs are already useful tools for drug development and modeling of diseases, and scientists hope to use them in transplantation medicine. Viruses are currently used to introduce the reprogramming factors into adult cells, and this process must be carefully controlled and tested before the technique can lead to useful treatments for humans.[45]

2.4 STEM CELL–NANOFIBER INTERACTIONS IN REGENERATIVE MEDICINE AND TISSUE ENGINEERING

Research in the area of drug delivery and tissue engineering witnessed huge progress because of their unlimited potential to improve human health. Drug delivery and tissue engineering are closely related fields, in which both drug delivery vehicles and tissue-engineered scaffolds need to be biodegradable and biocompatible. Controlled drug delivery strategies not only increase the efficacy of drugs but also maximize patient compliance, enhancing the ability to use poorly unstable/soluble and toxic drugs.[46] Such highly selective and effective therapeutic and diagnostic modalities can have a dramatic impact in medicine. Electrospun nanofibrous scaffolds were used as a carrier for both hydrophilic and hydrophobic drugs, in which the modulation of scaffold composition, morphology, and porosity are primarily carried out for a controlled drug release.[47] In tissue engineering approaches, it is important to recapitulate proper function and organization of native tissues as much as possible, which is usually done by mimicking tissue properties at nanoscale. ECM provides a natural web of tissue-specific and organized nanofibers support and maintains the cell microenvironment. Cells reside in a unique complex environment, and hence scaffolds for tissue engineering approaches maintain and regulate cell behavior[48] (Fig. 2.4). The design and fabrication of these substrates will require either a surface is naturally adhere to ECM molecules or that reproduces high-affinity binding sites for cell-associated receptors to reproduce the natural tissue organization observed in the pancreas, liver, and cartilage. Moreover, the utilization of electrospun nanofibrous scaffolds as cell delivery vehicles has been substantially increased in recent years owing, in part, to the physical similarities between nanofibrous scaffolds and ECM found in native tissues.[49] Such approaches might even be used to regulate and replicate *in vitro* cellular environment for stem cell differentiation.

2.4.1 Skin

Chronic wounds present a worldwide growing health and economic problem because of a steadily increasing number of patients, high morbidity and risk of amputations, unsatisfactory results of existing therapies, and heavy socioeconomic burden. Patients with 50% total body surface area (TBSA) full-thickness wounds have only 50% of undamaged skin left, which could be used for split-thickness skin harvesting. Donor sites would add to the total wound size, resulting in a wound area covering 100% of the body.[50] These donor sites heal with some scarring and may be very painful; hence, an

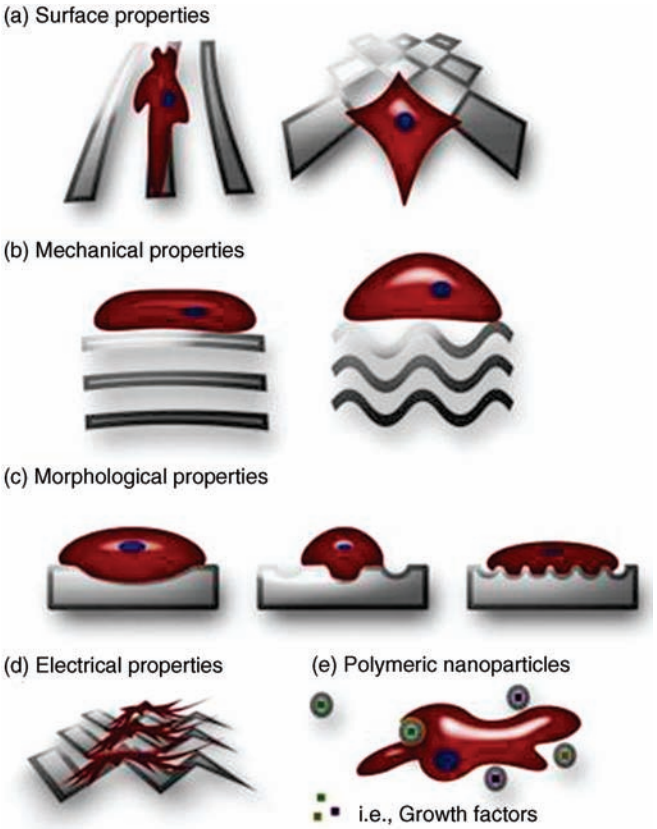


FIGURE 2.4 Scaffold properties. (a) Surface properties. The surface topography could drive cell adhesion, proliferation, migration, and differentiation. (b) Mechanical properties. Stem cells respond to the mechanical properties of the substrate on which they are growing, thus changing their fate. (c) Morphological properties. Scaffold morphologies for stem cell biomaterial interaction may vary in terms of interconnectivity, pore size, and shape. (d) Electrical properties. Electrical properties of the substrates are important issues in biomaterial–cell interaction. (e) Polymeric nanoparticles. Different smart nanosystems, nanoparticles, and nanoshells can be developed based on biodegradable polymers. Biodegradable nanosystems allow improvement of the therapeutic value of several water-soluble and nonsoluble bioactive molecules by improving bioavailability, solubility, and retention time. Reproduced with permission from Ref. [48].

additional analgesic pharmacological load is required for skin regeneration. Alternate lifesaving approaches in the treatment of extensive full-thickness wounds, in which donor sites for split-thickness skin grafts (SSG) harvesting are not available, include the use of cultured autologous keratinocytes, bioengineered skin substitutes, or both.[51] Significant progress has been made recently in the development and clinical use of these products. The most common skin injuries or skin wounds are categorized on the basis of the depth of the skin injury: epidermal or full-thickness skin wounds. Skin can

regenerate itself from minor epidermal injury. However, when the injury is a full-thickness skin wound (loss of both epidermis and dermis), the damaged skin cannot regenerate spontaneously. Natural repair of wound healing is slow compared with the rapid wound cover needed to reduce the time of wound healing.[52] Aligned and random fibrous scaffolds fabricated with fiber diameters down to 100 nm range with a wide range of pore sizes for the scaffolds.[53] These fiber mats have large surface areas available to interact with the cell surfaces and varying levels of porosity that enable differing amounts of cellular infiltration. Porosity and a ratio of high surface area to volume of the mats also facilitate diffusion into 3D structures, aiding in mass transfer. Nanofibrous scaffolds not only serve as carriers for the delivery of drugs but are also used as scaffolds for engineering skin, bone, cartilage, and vascular and neural tissue engineering.[54] Nanofibers can be electrospun in various patterns depending on the applications such as random, aligned, core shell, yarn, and fiber bundle. The mechanical properties of tissue engineering scaffolds are of the utmost importance in order for them to adequately perform their function. Various polymeric nanofibers have been investigated as a novel wound dressing material and as hemostatic devices. The high surface area of nanofiber matrix allows oxygen permeability and prevents fluid accumulation at the wound site. On the other hand, the small pore size of nanofibrous matrix efficiently prevents bacterial penetration, making them ideal candidates for wound dressings, where dressings for human wounds aimed to protect, removal of exudates, inhibition of exogenous microorganism invasion, and improved appearance. Systemic transplantation and local implantation of MSCs are promising treatment methods for skin wounds, especially for chronic wounds. The mechanisms by which BM-MSCs participate in cutaneous wound healing is by either differentiating into phenotypes of various damaged cells or by enhancing the repair process by creating a microenvironment that promotes the local regeneration of cells endogenous to the tissue. Nanofibrous scaffolds have been recently used in the field of tissue engineering because of their nanosize structure, which promotes cell attachment, function, proliferation, and infiltration. Recently, Wu et al. proved that BM-MSC-treated wounds exhibited significantly accelerated wound closure with increased reepithelialization, cellularity, and angiogenesis. Nanofibers have also been shown to enhance infiltration of stem cells.[55] Their results demonstrated that hMSCs isolated from human BM can differentiate into epithelial-like cells and may thus serve as a cell source for TE and cell therapy of epithelial tissue. Parenteau-Bareil et al. (2011) proved collagen–chitosan porous scaffolds mimicking the ECM of natural proteins for tissue engineering dermis.[56] To induce epithelial differentiation, they cultured MSCs using epidermal growth factor (EGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF)-II. Jin et al. (2011) showed the comparative scanning electron microscope (SEM) images of differentiated MSCs of epidermal phenotype and undifferentiated MSCs grown on collagen/poly(L-lactic acid)-*co*-poly (ε-caprolactone) (Coll/PLACL) nanofibrous scaffolds[57] (Fig. 2.5). SEM images of MSCs seeded with normal growth medium on the Coll/PLACL scaffold attached and remained undifferentiated with a fibroblastic phenotype. However, with time in culture, MSCs grown on Coll/PLACL nanofibrous scaffolds using epidermal induction medium acquired polygonal and round morphologies, and no cobblestone pattern clusters were

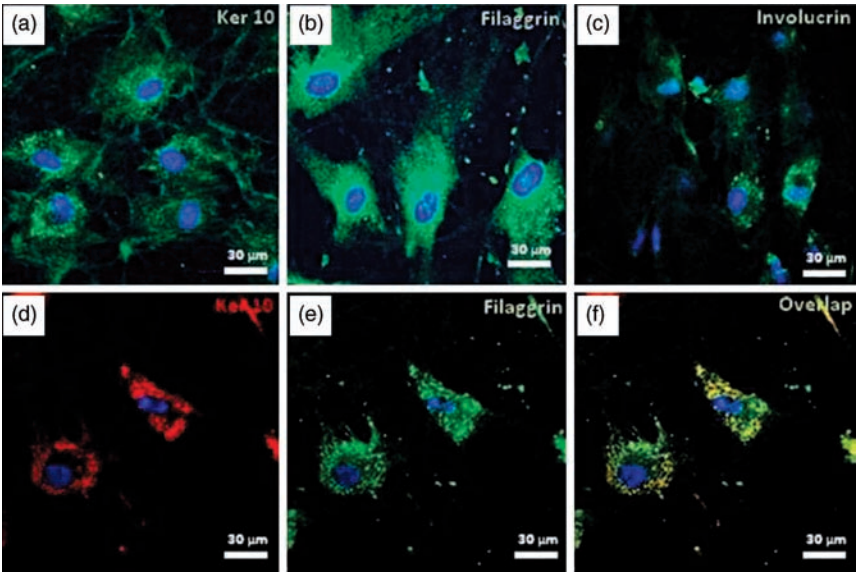


FIGURE 2.5 Laser scanning confocal microscope (LSCM) images of MSC grown in epidermal induction medium on Coll/PLLCL nanofibers expressing Ker 10 (a), filaggrin (b), and involucrin (c). Double staining for Ker 10 (d) and filaggrin (e) and (f) overlay image of (d) and (e) after 15 days of cell culture. Reproduced with permission from Ref. [57].

found on the Coll/PLACL nanofibrous scaffolds. The study suggest that the electrospun Coll/PLACL nanofibers supported the differentiation of MSCs in the presence of growth factors, thereby creating the possibility of cell–scaffold transplantation of a construct with differentiated keratinocytes to the sites of skin injury. Kobayashi and Spector (2009) investigated the clinical effects of mechanical stress on the behavior of BM-MSCs in a collagen type I/glycosaminoglycan scaffold matrix for 1 week under cyclic stretch loading conditions.[58] Their results suggested that mechanical stress may affect the proliferation and differentiation of stem cells and, subsequently, the wound healing process via interactions between the stem cells and scaffold matrix. Adipose-derived stem cells (ADSCs) secrete various growth factors that control and manage damaged neighboring cells, and this has been identified as an essential function of ADSCs. ADSCs stimulated both collagen synthesis and migration of dermal fibroblasts, which improved wrinkling and accelerated wound healing in animal models.[59,60]

Novel cost-effective electrospun nanofibrous scaffolds are established for wound dressing and allogeneic cultured dermal substitute through the cultivation of human dermal fibroblast for skin defects. A combination of growth factors together with the porous structure of the scaffolds might substantially improve the skin regeneration efficacy. This can be achieved by a simple incorporation of growth factors during the scaffold preparation, either with an electrospinning process or obtaining a controlled release of growth factors via a coaxial electrospinning technique.[61]

2.4.2 Cardiac

Myocardial infarction occurs when supply of oxygen and nutrients to the cardiac muscle is impaired, usually because of occluded coronary arteries. As a result, massive cell death occurs in the affected heart region.[62] Besides life-threatening arrhythmia, damage of muscle tissue in the left ventricle can cause dysfunction and remodeling in terms of progressive dilation, imparting structural changes that culminate in the formation of noncontractile fibrotic scar tissue.[63,64] Hence, the damage incurred to the heart wall is beyond recall because the myocardial tissue has limited regeneration capacity.[65,66] Although the body compensates for left ventricular (LV) remodeling initially, mismatch of mechanical and electrical properties of scar with native myocardium ultimately affects the functioning of the heart, leading to chronic heart failure, whereby the heart cannot pump adequate blood for all metabolic activities of the body.[67] Many intriguing modes of regenerating injured myocardium have emerged over time with pioneering research in a variety of technologies, including cell therapy using various cell types, injection of biomaterials, bioengineered patches, 3D construct implantation, and even bioreactor-treated implants.[68–70] In native tissue, cell growth and structural development is supported by the ECM. Lack of an appropriate microenvironment in scarred myocardium might be a plausible reason for the colossal loss and ineffective homing of injected cells. To enhance cell attachment, proliferation, and differentiation, it is necessary to mimic some of the nanostructure of the natural ECM. Scaffolds with nanoscaled architecture provide larger surface area to adsorb proteins and provide more binding site to cell membrane receptors, unlike microscale and flat surfaces.[71] This makes nanofabrication of biomaterials for myocardial regeneration is an attractive strategy. Traditionally, a cardiomyocyte has been considered terminally differentiated in response to injury. However, recent evidence raises the possibility that a natural system of myocyte repair exists. According to this study, fewer than 50% of cardiomyocytes are exchanged during a normal life span. This system appears to be inadequate in face of an ischemic or heart failure insult and its treatment.[12] Ultrafine woven nanofibers having ECM-like topography can be achieved by electrospinning of biomaterial or self-assembly of certain peptides via noncovalent interactions.[72,73] A versatile, biodegradable *in vitro* construct made of poly(ε-caprolactone) (PCL) nanofibers and cardiomyocytes was reported by Shin et al. (2004).[74] Being able to foster cellular ingrowth, it was proposed to be more desirable than 3D construct in patch application.[74] The bioengineered cardiac tissue structure and function, chemistry, and geometry of the provided nano- and microtextured using poly(lactic-*co*-glycolic acid) (PLGA) nanofibers were later demonstrated. Thereafter, nanofibers of blended and conductive polymers were shown to be potential choices in MTE.[75,76] Recently, coaxial electrospun poly(glycerol–sebacate) (PGS) nanofibers were fabricated, opening up new horizons in MTE owing to its resemblance to elastin fibers.[77] Recently, Mukherjee et al. showed that suitable cell–material interactions on the nanoscale can stipulate organization on the tissue level and yield novel insights into cell therapeutic science while providing materials for tissue regeneration.[78] Inspired by microscopic analysis of the ventricular organization

in native tissue, we fabricated a scalable, nanotopographically controlled *in vitro* model of nanoscale poly(L-lactic acid)-*co*-poly(ε-caprolactone)/collagen biocomposite scaffold of nanofibers measuring 594 ± 56 nm to mimic the native myocardial environment for freshly isolated cardiomyocytes from rabbit heart and specifically underlying ECM architecture to address specificity of underlying matrix in overcoming challenges faced by cellular therapeutics. Guided by nanoscale mechanical cues provided by the underlying random nanofibrous scaffold, the tissue constructs displayed anisotropic rearrangement of cells, characteristic of the native cardiac tissue. Surprisingly, cell morphology and growth and the expression of an interactive healthy cardiac cell population were exquisitely sensitive to differences in the composition of nanoscale scaffolds that features of the surrounding ECM.[79] Ravichandran et al. fabricated PGS/gelatin core/shell fibers and gelatin fibers alone by electrospinning for cardiac tissue engineering. In these PGS/gelatin core/shell fibers, PGS used as core polymer to impart the mechanical properties and gelatin as a shell material to achieve

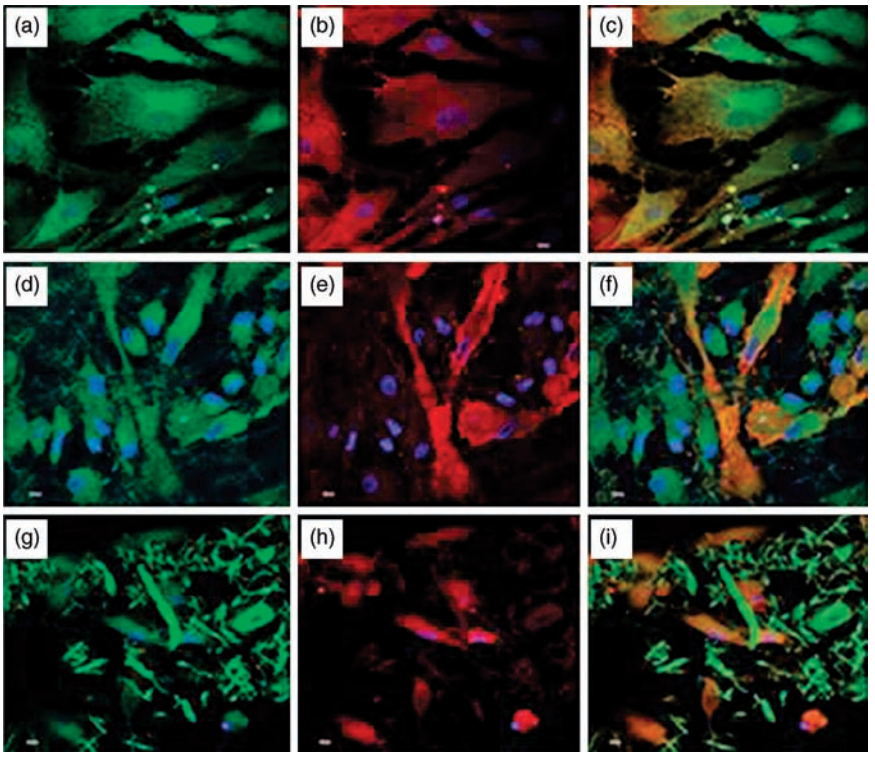


FIGURE 2.6 Core/shell (PGS/gelatin) fibrous structure for regeneration of myocardial infarction. Dual immunocytochemical analysis for the expression of MSC marker protein CD 105 (a, d, g) and cardiac marker protein actinin (b, e, h) in the coculture samples and the merged image showing the dual expression of both CD 105 and actinin (c, f, i); on the TCP (a, b, c), gelatin nanofibers (d, e, f), and PGS/gelatin core/shell fibers (g, h, i) at 60× magnification. Nucleus stained with DAPI. Reproduced with permission from Ref. [80].

favorable cell adhesion and proliferation. The study demonstrated that PGS/gelatin core/shell fibers, having good potential biocompatibility and mechanical properties for fabricating nanofibrous cardiac patch, favor differentiation MSC into the cardiac lineage[80] (Fig. 2.6). It is likely that the structure and function of the *in vivo* cardiac tissue are regulated by much smaller nanoscale cues provided by the ECM, which is responsible for extensive control over cell and tissue function.[81] Thus, biomaterials with controlled bioactivity could be potentially designed to respond and enhance the regenerative capability of myocytes or exogenous cells to adjust the myocardial mechanical load for myocardial tissue engineering.

2.4.3 Bone and Cartilage

Bone and cartilage tissue regeneration remains an important challenge in the fields of orthopedic and craniofacial surgery. Every year, millions of people around the world have bone defects arising from trauma, tumors, biochemical disorders, and abnormal skeletal development; the worst scenario is that many die because of insufficient bone and cartilage replacements.[82] Cell-based therapies such as autologous chondrocyte transplantation (ACT) has been used clinically since 1987 to treat full chondral thickness defects. Nearly 12,000 patients with full chondral thickness defects have benefited from ACT worldwide.[4] Currently, more than 250,000 knee and hip replacements are performed in the United States each year for end-stage disease joint failure, and many other patients have less severe cartilage damage.[83] The emerging trend in recent decades is the use of nanofibrous scaffolds as synthetic ECM with which cells interact before forming a new tissue. These nanofibrous scaffolds are capable of providing the desired support needed for cell adhesion, proliferation, and differentiation.[84] The osteoinductive and osteoconductive properties that are vital for mineralization and bone growth, various kind of material used for the preparation of scaffolds. The scaffold should be biocompatible and biodegradable, and the rate of biodegradation should match the rate of formation of the new tissues. It should be highly porous and should allow nutrient transport and tissue ingrowth. Several cell types have been reported for increased proliferative ability on nanofibrous scaffolds than control tissue culture plate (TCP). Osteoblasts, when seeded on nanofibrous scaffolds, have shown increased proliferation within 7–12 days of culture[85] because an increase in proliferation reduces the scar tissue formation, which eventually reduces the surgical necessities to remove scar tissue. Nanofibers enhanced the proliferation and differentiation of many cell types, including neural progenitors,[86] hepatocytes,[87] and osteoblasts.[88] Nanofibrous scaffolds also have the ability to rescue cells from regression, promoting them to a more immature phenotype during expansion culture.[89] The key attachment proteins such as fibronectin, vitronectin, and laminin have been found to adsorb to nanofibrous scaffolds 2.6–3.9 times higher than the solid-walled (SW) scaffolds.[90] A variety of natural and synthetic biodegradable materials have been used for the fabrication of nanofibrous scaffolds in tissue engineering, but the main disadvantage in these synthetic scaffolds is the lack of biological recognition sites on their surface; in other words, they are noninformational scaffolds. Various groups have tried to modify the

surface of scaffolds to increase cell–surface interactions, eventually increasing the rate of mineralization.[91,92] Human ESC-derived embryoid body cells were cultured in the presence of osteogenic supplements such as ascorbic acid and beta-glycerophosphate (BGP) for 14 days, and dexamethasone was added to this medium for another 24 h. The stimulated cells were further seeded onto poly(lactic acid) (PLA) scaffolds and implanted subcutaneously to the back of immunodeficient mice for 5 weeks. Discrete areas of mineralization were observed, and osteocalcin was expressed by the implanted cells.[93] The cell–cell interactions and bone morphogenic proteins secreted by primary bone-derived cells stimulated human embryonic stem cells (hESCs) into osteogenic lineages in a direct coculture system.[94] Cell extracts derived from hESC-derived osteogenic cultures induced undifferentiated hESCs into osteogenic lineage.[95] Electrospun nanofibrous scaffolds have been successful in supporting the maintenance of chondrocyte phenotype and chondrogenic induction of stem cells.[96] These nanofibrous scaffolds have given hope for cartilage tissue-engineering applications. Chondrocytes seeded on electrospun scaffolds have shown increased proliferation within 3 weeks of culture than the controls. Increased chondrocytes proliferation, differentiation, and attachment have been studied in nanofibrous scaffold by Li et al.[97] The differentiation of stem cells to chondrocytes on nanofibrous scaffold was comparable to an established cell pellet culture. It was advantageous to use nanofibers rather than a cell pellet system, owing to their better mechanical properties, oxygen–nutrient exchange, and ease of fabrication. Cheng et al. reported that human cartilage cells attached and proliferated on hyaluronic acid nanocrystals homogeneously dispersed in PLA and that collagen fibers of 110–1.8 mm diameter supported chondrocyte growth and infiltration.[98] Chondrogenesis of MSCs was supported on 3D porous aqueous-derived silk scaffolds, forming cartilage-like tissue with spatial distribution of cells and ECM, with expression of chondrogenic genes, and zonal architecture resembling the native

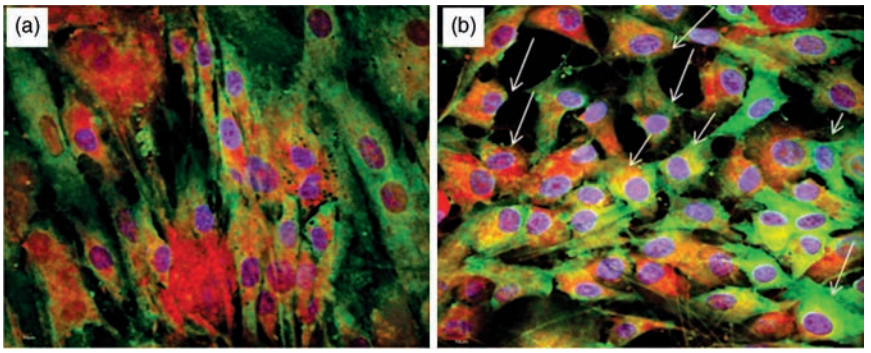


FIGURE 2.7 Confocal microscopy image of PLLA nanofibers (a) and PLLA/PBLG/Col/n-HA nanofibers (b) showing dual expression of both ADSC specific marker protein CD 105 and osteoblasts specific marker protein osteocalcin. *Arrows* indicate the characteristic cuboidal morphology of osteoblasts shown by the ADSCs that have undergone osteogenic differentiation on the PLLA/PBLG/Col/n-HA nanofibers at 60× magnification. Reproduced with permission from Ref. [102].

tissue.[99,100] Chondrogenesis was improved in silk scaffolds compared with collagen scaffolds in terms of cell attachment, metabolic activity, proliferation, ECM deposition, and glycosaminoglycan (GAG) content.[101] However, the biggest challenge with using nanofibrous scaffolds is the intrinsically small pore size of the fibers, which limits infiltration and migration of the seeded cells and affects cell distribution in the scaffold. This limitation can be overcome by changing the cell-seeding procedures on the scaffold for cartilage. Smart materials like PLLA/PBLG/Col/n-HA scaffolds elicit therapeutic effects by incorporating bio-signaling molecules within the nanofibers, such as proteins and genes, hold great promise as scaffolds for bone tissue engineering with drug delivery applications (Fig. 2.7).

2.4.4 Neural

Neural diseases represent a very complicated and significant clinical problem; for example, in the United States alone, about 250,000–400,000 people are living with spinal cord injury, and nearly 13,000 additional people sustain spinal cord injuries each year. Peripheral nerve lesions are serious injuries, affecting 2.8% of trauma patients annually, leading to lifelong disability.[103] Allograft and xenografts have certain disadvantages such as disease transmission and immunogenicity. The other disadvantages of autograft nerve repair systems include the loss of function at the donor nerve graft site and mismatch of damaged nerve and graft dimensions. TE offers promising strategies and provides viable alternatives to surgical procedures for harvested tissues and implants.[104] Many researchers have attempted to regenerate nerve tissue by combining scaffolds with MSCs, and it has also been shown that the chemical composition of scaffolds influences the differentiation of MSC to nerve cells. Prabhakaran et al. compared the potential of hMSCs for *in vitro* neuronal differentiation on poly(L-lactic acid)-*co*-poly(ε-caprolactone)/collagen (PLCL/collagen) and PLCL nanofibrous scaffolds. Many researchers have attempted to regenerate nerve tissue by combining scaffolds with MSCs, and it has also been shown that the chemical composition of scaffolds influences the differentiation of MSC to nerve cells.[105] Prabhakaran et al. compared the potential of hMSCs for *in vitro* neuronal differentiation on PLCL/collagen and PLCL nanofibrous scaffolds.[106] MSCs have been shown to have an important regenerative potential after transplantation into the stumps of transected sciatic nerves. Lopes et al. evaluated the regeneration of peripheral nerve using a tubular nerve guide of resorbable collagen filled with MSCs. Their results showed that a biodegradable collagen tube filled with MSCs induced better regeneration of peripheral nerve fibers across a nerve gap than a collagen tube without cells.[107] Oliveira et al. fabricated PCL conduits for regeneration of transected mouse median nerves and investigated the effect of MSCs on nerve regeneration by seeding MSCs on PCL nerve conduit before grafting of PCL conduits.[108] Hou et al. differentiated MSCs into cells expressing characteristic markers of Schwann cells and used PLGA nerve conduit along with differentiated MSCs for bridging a 10 mm long sciatic nerve defect.[109] Lee et al. constructed nanoscale ridge/groove pattern arrays using UV-assisted capillary force lithography on polyurethane acrylate (PUA) and showed that the nanoscale ridge/groove pattern

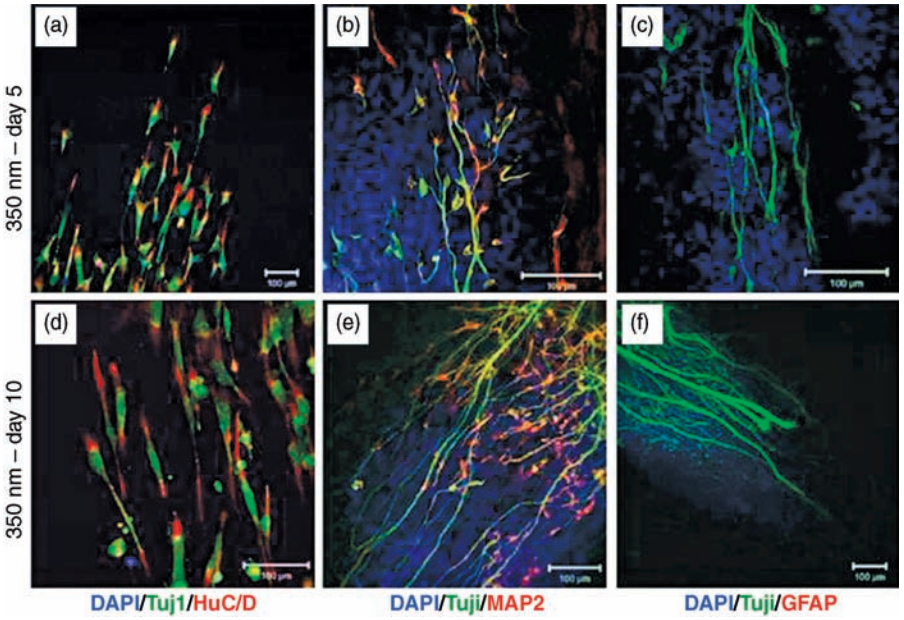


FIGURE 2.8 Immunofluorescence staining of human embryonic stem cells (hESCs) with neural and glial markers. (a, d) hESCs were immunolabeled for DAPI, Tuj1, and HuC/D. (b, e) hESCs were immunolabeled for DAPI, Tuj1, and MAP2. (c, f) hESCs were immunolabeled for DAPI, Tuj1, and GFAP. hESCs cultured for 5 days (a, b, c) and 10 days (d, e, f) on the 350 nm ridge/groove pattern arrays. Reproduced with permission from Ref. [110].

arrays can rapidly and efficiently induce the differentiation of hESCs into neuronal lineages even in the absence of differentiation-inducing agents[110] (Fig. 2.8). Functionalizing biomaterials with bioactive molecules such as ECM-derived cell adhesive molecules to impregnate guiding cues on the scaffolds is an emerging research interest and can provide an instructive extracellular microenvironment for neural regeneration.

2.5 CONCLUSIONS

Nanotechnology has the potential to change medical research dramatically with advances in cell-based technologies. Tissue engineering is the promising therapeutic approach that combines cells, biomaterials, and microenvironmental factors to induce differentiation signals into surgically transplantable formats and promote tissue repair, functional restoration, or both. One obstacle can be identified as the scaffolds play an important role as the ECM, but they are often unable to create the exact or correct microenvironment during the engineered tissue development to promote the accurate *in vitro* tissue development. The emerging and promising next generation of engineered tissues relies on producing scaffolds with an informational

function, such as material containing a growth factors sequence that facilitates cell attachment, proliferation, and differentiation that is far better than noninformational polymers. Stem cell-based tissue engineering has matured from its original goal of prolonging or replacing; it now involves customized systems are designed to achieve specific spatial and temporal control in tissue engineering applications. The new generation of tissue engineering systems incorporates "smart" biosensing functionalities and will enable unaided *in vivo* feedback control. To advance the biotechnological and especially biomedical nanotechnology applications of polymer nanofibers from the perspective to commercialized stages, collaborative interdisciplinary research involving surgeons, material scientists, biologist, physiologists, clinicians, and engineers is required. One may believe that continual research and development in this field not only shortens the distance to a practical application in the listed areas but also open up other new opportunities for polymer nanofibers in drug delivery and tissue engineering applications.

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3

MICRO- AND NANOENGINEERING APPROACHES TO DEVELOPING GRADIENT BIOMATERIALS SUITABLE FOR INTERFACE TISSUE ENGINEERING

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3.1 INTRODUCTION

Interface tissue engineering (ITE) is a rapidly developing field that focuses on the fabrication and development of interfacial tissues for regenerative applications. Interfacial tissues in the human body are primarily found at the interface between

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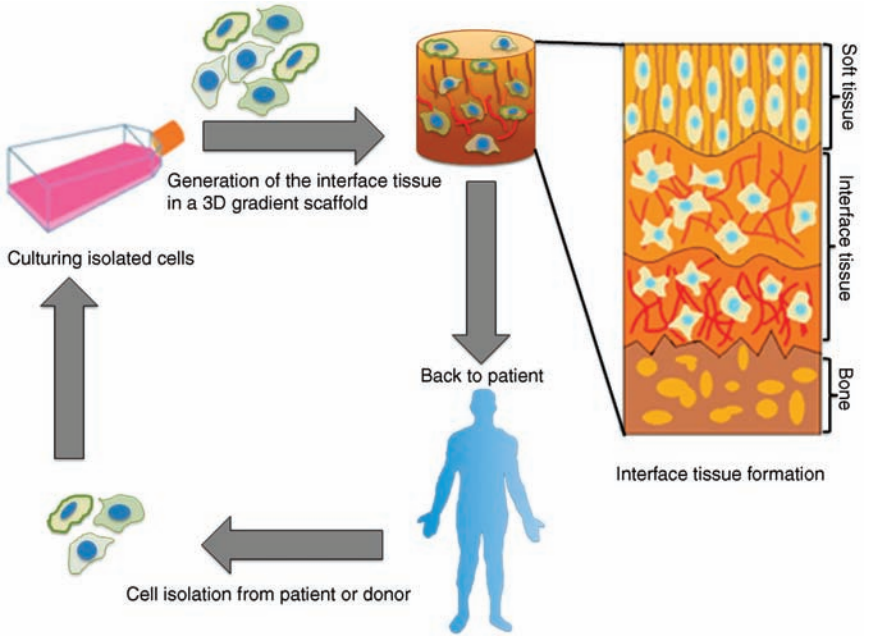

FIGURE 3.1 The concept of interface tissue engineering. 3D = three-dimensional. Modified from Ref. [1] with permission from Elsevier.

soft and hard tissue regions, such as cartilage-to-bone, tendon-to-bone, ligament-to-bone, and other tissue interfaces (e.g., dentin-to-enamel). Tissue engineering (TE) often uses conventional biomaterials to engineer homotypic tissues such as skin, nerve, cartilage, and bone. However, interface tissues are specialized tissues that consist of complex structures with anisotropic properties; thus, conventional biomaterials made of either monophasic or composite materials are inefficient in facilitating interface tissue formation. To engineer an interfacial tissue, biomaterials with a precise distribution of spatial and temporal properties, heterotypic cells, and signaling molecules are required. Therefore, gradient biomaterials with anisotropic properties are more appropriate for ITE studies than their conventional counterparts and may provide a better cellular microenvironment for the support and culture of heterotypic cell populations to generate functional tissue interfaces. A typical ITE process involving gradient biomaterials is schematically illustrated in Figure 3.1.

The development of gradient biomaterials is one of the main criteria for successful ITE development. These gradient biomaterials serve as the extracellular matrix (ECM), providing cells with a temporary structural support to grow and organize into functional tissues.[1] Indeed, the native ECM within tissue interface regions is composed of several biophysical and biochemical cues, which often exist along spatial and temporal gradients. These cues regulate most cell behaviors, such as alignment, motility, differentiation, and mitosis, which assist critical biological processes such as the immune response, embryogenesis, and interface tissue formation. Therefore, synthetic scaffolds made of gradient biomaterials have numerous

advantages over their conventional monophasic counterparts in the context of the material's structure and function for the purpose of interface tissue regeneration. However, the preparation and characterization of gradient biomaterials are generally more difficult than those of homogenous biomaterials because of their complex arrangement and design. Recent advances in micro- and nanoengineering approaches have enabled the development of biomaterials or synthetic scaffolding systems with gradients in material properties that favor the culture and growth of heterotypic cells, particularly with regard to cell differentiation, which is a necessary step toward the development of tissues suitable for ITE.

With these previous findings as a foundation, this chapter discusses various techniques used in the fabrication of gradient biomaterials or scaffolds suitable for engineering tissue interfaces and how the gradient features of the biomaterials influence cellular behaviors such as adhesion, migration, differentiation, and heterotypic interactions during tissue organization. In addition, an overview of various gradient biomaterials and their physical, chemical, and biological classifications is provided. Finally, potential challenges and future directions of the emerging field of ITE are discussed.

3.2 CLASSIFICATION OF GRADIENT BIOMATERIALS

Gradient biomaterials are those with anisotropic properties. Such anisotropies can be observed in the material composition (e.g., different polymer concentrations or compositions), the material structures (e.g., gradients of thickness or porosity), the physical and mechanical properties of the material (e.g., gradients of wettability or stiffness), and the interactions of the material with cells (e.g., cross-gradients of adhesive and nonadhesive polymers). In addition, anisotropies can be added to the material by coating (e.g., gradients of adhesion peptides) or by incorporating a soluble or immobilized molecular factor or drug into the biomaterial.[2] Figure 3.2 presents examples of different types of gradients created in the composition and structure of materials, including gradients in chemical composition, thickness, and porosity.

Gradient biomaterials have recently been used in the field of tissue engineering, and their development for biomedical applications has just begun. Table 3.1 summarizes some gradient types investigated in cell studies. Gradient biomaterials can be generally classified into three types depending on their physical, chemical, and biological properties, which are further discussed below.

3.2.1 Physical Gradients

Biomaterials with physical gradients are referred to as materials with a graded variation in their physical properties, including porosity, stiffness, and topography. Physical gradients are ubiquitous in the human body. Notable examples include bone structure and soft-to-hard interface tissues, such as ligament-to-bone, cartilage-to-bone, or tendon-to-bone interfaces. These interfaces convert the mechanical properties of one tissue into the mechanical properties of the other tissue via a gradual

TABLE 3.1 Gradient Types Used in Cell Studies and Tissue Engineering

Gradients	Materials Used	Applications	References
Physical			
Porosity	Agarose/gelatin gel, polyacrylamide gel	Electrophoresis, bone tissue engineering	[3,4]
Mechanical properties	PLGA nanofiber, agarose gel, polyacrylamide gel	Cell migration, differentiation, tendon-to-bone ITE	[5,6]
Chemical			
Composition	PLGA nanofiber/ hydroxyapatite (HA), collagen/HA	Scaffolds with a gradient of mineralization for ITE	[5,7]
Biological			
Soluble molecules	Poly(2-hydroxyethyl methacrylate) (p(HEMA) gel, polyacrylamide-based gel	Cell attachment and migration; cell proliferation and differentiation; tissue engineering, axonal guidance	[8]
Immobilized molecules	Polyethylene glycol (PEG) gel, agarose gel	Cell adhesion and alignment; cell migration, neurite outgrowth, tissue engineering	[9,10]

ITE = interface tissue engineering.
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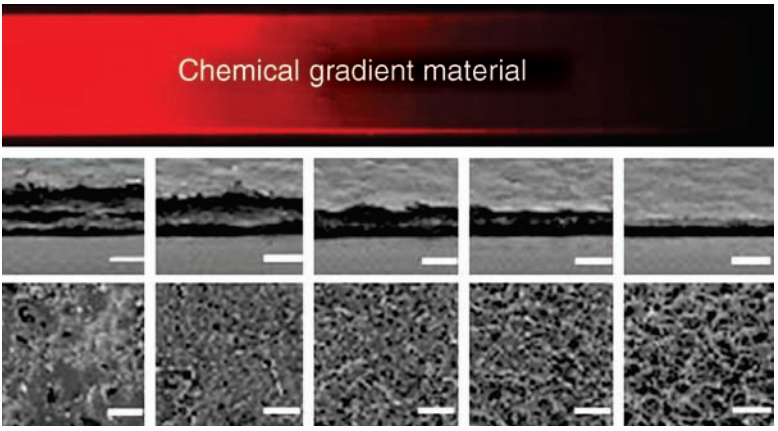

FIGURE 3.2 Poly(ethylene glycol)–diacrylate hydrogel in a microchannel with a chemical composition gradient and its correlated thickness and porosity (scale bar: 50 μm). Adapted from Ref. [1] with permission from John Wiley & Sons.

change in the structural organization and nature of the tissue. Interfacial tissues are complex structures with heterotypic cells surrounded by subtle variations in the ECM, which contains physical, chemical, and mechanical cues. Therefore, scaffolds with graded physical properties are better suited to promoting interface reconstruction.

In tissue engineering, the most frequently used physical gradients are porosity gradients, stiffness gradients, and surface gradients. Porous scaffolds fabricated from biomaterials have also been widely used in tissue reconstruction. In fact, scaffolds with appropriate porosities and interconnected pores with different size ranges are typically required to facilitate cell infiltration and other essential cellular functions. A good example of a physical porosity gradient in the native body is the interface between the cortical and trabecular bone regions, which exhibits a smooth and continuous transition from low porosity at the cortical bone region to high porosity at the trabecular bone region.[11] Porosity and pore size are very important features of a tissue scaffold that greatly affect cell behaviors, particularly cell adhesion, migration, proliferation, and phenotype expression.[12] For example, whereas endothelial cells showed the highest proliferation and ECM production profiles when cultured on scaffolds with a 5 μm pore size compared with scaffolds with larger pore sizes, hepatocytes preferred 20 μm, fibroblasts 90–360 μm, and osteoblasts 100–350 μm pore sizes.[13,14] Consequently, when cells are cultured on a scaffold that has a gradient of porosity or pore size, they tend to preferentially colonize in some areas rather than others. For example, cells from a mixture of chondrocytes, osteoblasts, and fibroblasts cultured on a pore-size gradient colonized in different areas depending on the size of the pores.[15] Chondrocytes and osteoblasts grew well on the larger pore size area, whereas fibroblasts preferred the smaller pore size area. Woodfield et al. showed that a pore size gradient from 200 to 1650 μm promoted an anisotropic bovine chondrocyte cell distribution and anisotropic glycosaminoglycan (GAG) deposition.[16] This anisotropic cell distribution caused by a gradient in porosity or pore size can be used to investigate the interactions of the cells with the scaffold, control cell migration and proliferation, guide tissue ingrowth, or mimic a physiological interface.

Biomaterials with gradients in mechanical properties are often used to engineer interfacial tissues. A good example of a biomechanical gradient in the body is the tendon-to-bone interface, where the stiffness of the bone gradually converts to the elasticity of the ligament.[17,18] In tissue engineering, it is important that a scaffold matches the mechanical properties of the host tissue. For example, in bone regeneration, if the scaffold has lower mechanical properties than the bone itself, the scaffold will not be able to withstand the physiological load and may break. In contrast, if the scaffold has higher mechanical properties than the bone, it will shield the bone from the load and thus may cause bone resorption (stress-shielding effect). A great deal of research is underway to mimic the mechanical properties of bone using gradient biomaterials.

Material stiffness is another key property that affects cell behaviors, notably cell spreading, proliferation, and differentiation. This importance of stiffness exists because cells can precisely sense physical stress and adjust the rigidity of their

cytoskeleton, as their traction force at their anchoring site.[19,20] For example, Kloxin et al. reported the effect of poly(ethylene glycol) (PEG) films on valvular interstitial cells (VICs) with a gradient of elastic modulus ranging from 7 to 32 kPa.[21] These authors observed a graded differentiation of VICs into myofibroblasts, which increased with an increase in the elastic modulus. Decreasing the elastic modulus to 7 kPa led to a reversal of differentiation from myofibroblasts to VICs. Other studies have demonstrated that fibroblasts or smooth muscle cells migrate from softer areas to stiffer areas when cultured on a stiffness gradient.[22] Each cell type responds to stiffness in a different fashion. For example, fibroblasts grow well on stiff materials with a Young's modulus of 34 kPa; neurons prefer soft materials with a Young's modulus of 50 Pa; and smooth muscle cells grow better on materials of moderate stiffness, with a Young's modulus of 8–10 kPa.[23,24] These experimental data show that cells respond strongly to biomaterials with physical gradients.

The surface properties of biomaterials also greatly affect cell behaviors. Surface gradients in terms of roughness, hydrophilicity, and crystallinity have a strong effect on cellular adhesion, spreading, proliferation, and ECM deposition. Washburn et al. introduced a roughness gradient from 0.5 to 13 nm on a poly(L-lactic acid) film and studied the effect of surface roughness with preosteoblastic MC3T3-E1 cells.[25] This study demonstrated that cells responded to roughness and that the cell density decreased with increasing roughness. To investigate the effect of surface features on cellular functions, Meredith et al. used combinatorial methods.[26] These authors fabricated poly(D,L-lactide) (PD)/poly(ε-caprolactone) (PCL) blend libraries with gradients in composition, annealing temperature, and surface structure and loaded them with MC3T3-E1 cells to probe the effects of different surface properties on the cell responses. The experimental data from various studies demonstrate that the cells are highly sensitive to surface features, which dramatically affect cell functionality, shape, size, and regulatory pathways. Therefore, the physical properties of biomaterials must be taken into consideration when designing biomimetic gradient biomaterials for tissue engineering and other biomedical applications.

3.2.2 Chemical Gradients

Biomaterials with chemical gradients are referred here to as materials with gradients of chemical functionalities or properties. This type of gradient biomaterial can be obtained by changing the chemical functionality of the substrate by physical adsorption or chemical bonding. For example, the chemical functionalities of a biomaterial can be changed by treating its surface with plasma or by grafting with chemical functional groups.[27,28] Chemical gradients can also be observed in terms of the material composition of a biomaterial. For example, hydrogels can be polymerized with different material compositions by establishing a prepolymer concentration gradient before cross-linking or by varying the amount of ultraviolet (UV) irradiation during cross-linking.[29] Burdick et al. generated a hydrogel with a gradient of cross-linking densities by using two different poly(ethylene glycol)–diacrylate macromers (10 wt% PEG4000DA and 50 wt% PEG1000DA).[30] After photopolymerization, the 10 wt% macromer solution produced a thin network with a large mesh, whereas the

50 wt% macromer solution produced a larger network with a thin mesh, and the hydrogel presented a thickness gradient from 10 to 50 μm. This alteration results in a hydrogel biomaterial with a gradient in material composition.

The surface chemistry of biomaterials can also be covalently modified by spacers or other functional groups. A classical example is the use of self-assembled monolayers (SAMs). Liu et al. formed a gradient of $C_{11}OH$ SAMs on a gold layer substrate using electrochemical desorption, backfilled the spaces with $C_{15}COOH$, and then activated the carboxyl groups to fix adhesive protein molecules such as fibronectin (FN) or growth factors such as vascular endothelial cell growth factor (VEGF).[31] The cells moved faster toward the protein gradient when the graded surfaces were loaded with bovine aortic endothelial cells (BAECs) compared with the uniform control surface, and the effects of multiple gradients were cumulative. Surface changes have also been applied to obtain a gradient of wettability. For example, Yu et al. generated a gradient from superhydrophobicity to superhydrophilicity on rough gold surfaces via SAM formation.[32] Many of these studies with wettability gradients have focused on cell adhesion and spreading.[33,34] In some cases, a spacer has been used between the substrate and the active molecules or proteins that form the gradient. Mougin et al. generated a PEG gradient by diffusing a PEG–NHS solution through a gel layer coating a cystamine-modified gold surface.[35] When used in cell culture with bovine arteriole endothelium cells (BAVEC-1), a gradient of cell density was observed in the opposite direction of the increasing PEG concentration. These experimental data and others highlight the efficacy of biomaterials with chemical gradients in cell engineering.

3.2.3 Biological Gradients

Biomaterials with biological gradients have gradients of biological moieties such as proteins or biological molecules. Such gradients have been generated using immobilized or soluble forms. Examples include the generation of gradients with adhesive peptides and natural ECM proteins to study the cellular functions and improve biomaterial properties. The cellular response to a concentration gradient has been shown to be much stronger than the response to a single homogenous concentration exposure.[36] Moreover, Wang et al. have shown with human mesenchymal stem cells (hMSCs) that the cellular response can differ along a concentration gradient, as evidenced by the differentiation of stem cells into osteogenic and chondrogenic lineages along a bone morphogenic protein-2 (rhBMP-2) concentration gradient.[37] Different approaches can be used to expose cells to biological gradients.[38] For example, a biological moiety can be grafted onto the surface of a substrate or immobilized in a polymer matrix[9,39,40] or included as a soluble factor in the polymer matrix.[8,41] Similarly, cells can be encapsulated with the gradient in the polymer matrix[42] or attached to the substrate surface.[39] The arginine–glycine–aspartic acid (RGD) motif is a sequence found in native ECM proteins, such as fibronectin, fibrinogen, and laminin, that acts as a cell-adhesion ligand with integrins. RGD is often used to enable cell attachment to polymers such as PEG, which repel cells. NIH3T3 fibroblasts cultured on a PEG hydrogel with an immobilized RGD gradient

aligned and moved along the gradient.[5] In an interesting study, DeLong et al. cultured vascular smooth muscle cells (SMCs) on a gradient of basic fibroblast growth factor (bFGF) immobilized on a PEG substrate with RGD adhesion sites and showed cell migration along the gradient.[10] Jiang et al. have proposed a general method for immobilizing biomolecular gradients on a surface through the use of avidin–biotin bonding.[43] These researchers first immobilized a gradient of avidin on a surface by adsorption and then added a biotinylated molecule of interest. Knapp et al. used a chamber filled with collagen or fibrin gel that was divided into two parts by a Teflon barrier.[44]Human foreskin fibroblast (HFF) cells were encapsulated in one gel part; the other part contained the soluble fibronectin peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP), which is a fibroblast chemotactic factor. When the Teflon barrier was removed, the peptide diffused into the second gel part, forming a gradient of GRGDSP that induced the alignment and migration of the fibroblasts toward the region of higher peptide concentration. Other examples of morphogen gradients include those related to angiogenesis and axonal growth. PC12 neurites were promoted and guided when cultured in a nerve growth factor (NGF) gradient immobilized on poly(2-hydroxyethyl methacrylate) (p(HEMA)) or when cultured in a p(HEMA)/poly-L-lysine (PLL) scaffold loaded with NGF and neurotrophin-3 (NT-3) concentration gradients.[45,46] Similarly, primary fetal neural stem cells (NSCs) showed a rapid induction of glial fibrillary acidic protein (GFAP) when cultured on a hydrogel with a ciliary neurotrophic factor (CNTF) gradient.[47] In the same context, endothelial cells migrated along a surface density gradient of VEGF and formed sprouting elements when exposed to a VEGF gradient in a collagen gel.[31,48] Biological gradients have also been used to study cell metastasis in cancer.[49] Another dynamic research area involving morphogen gradients focuses on bone and cartilage engineering. He et al. have shown that RGD and BMP peptides found in bone morphogenetic protein-2 (BMP-2) acted synergistically when grafted onto a hydrogel to induce bone marrow stromal (BMS) cell osteogenesis and mineralization.[50] Cooper et al. printed a BMP-2 gradient on a DermaMatrix scaffold to demonstrate the spatial control of osteoblast differentiation.[51] Dormer et al. used poly(lactic-*co*-glycolic acid) (PLGA) microspheres loaded with BMP-2 or transforming growth factor beta-1 (TGFβ1) to generate a three-dimensional (3D) scaffold with cross-gradients of those biomolecules.[52] When loaded with human umbilical cord mesenchymal stromal cells (hUCMSCs) or human bone marrow stromal cells (hBMSCs), these gradient scaffolds exhibited spatial and temporal control of the protein release with ECM formation, glycosaminoglycan production, or alkaline phosphatase activity along the increasing concentration gradient. These and other experimental studies have demonstrated that the influence of biological gradients regulates cell behavior.

3.3 MICRO- AND NANOENGINEERING TECHNIQUES FOR FABRICATING GRADIENT BIOMATERIALS

An ideal tissue engineering scaffold should mimic the structure and function of native ECM, in which cells and tissue are organized into 3D architectures and are

triggered by a variety of signaling cues to support cell adhesion, proliferation, and differentiation. Numerous techniques have been used to fabricate materials that are suitable as tissue scaffolds. The next sections discuss the micro- and nanotechnologies that are widely used to fabricate gradient biomaterials.

3.3.1 Salt Leaching

Salt leaching is a popular technique that has been widely used to build scaffolds for tissue engineering, in which salt is used to create the pores or channels in the 3D polymeric scaffolds.[53] This technique involves finely crushing a salt and screening the particles of the desired size followed by casting the mixture of the polymer, salt, and organic solvent into a mold. After solvent evaporation, the salt particles are leached away with water to generate a porous scaffold. Salt leaching is a simple technique for fabricating porous polymeric scaffolds with controlled porosity and pore sizes, which can be achieved by controlling the amount of salt added and the size of the salt particles, respectively. This technique enables the building of materials with a high porosity, up to 92–98%, and with pore sizes ranging from 100 to 700 μm.[54,55] 3D porous scaffolds are used in tissue engineering to support cell attachment, proliferation, infiltration, nutrient transport, and waste removal.[56] However, to build a tissue, the choice of porosity and pore size depends on the cell-type chosen for a specific tissue application. For example, a 5 μm pore size appears optimized for neovascularization, 5–15 μm for fibroblast growth, 20 μm for hepatocytes, 20–125 μm for skin regeneration, 70–120 μm for chondrocytes, 45–150 μm for liver tissue growth, 60–150 μm for vascular smooth muscle adhesion, 100–300 μm for bladder smooth muscle cells, 100–400 μm for bone tissue growth, and 200–350 μm for osteoconduction.[57,58] Therefore, controlling the porosity, pore size, and pore morphology by the porogen[59] is important for the characteristics of a tissue scaffold. At low porosity, with a porogen volume of 65% or less, the number of contact points between particles decreases, leading to incomplete pore interconnectivity and porogen entrapment inside the scaffold.[60] Depending on the porosity and the nature of the porogen, the removal of porogen from the scaffold can be difficult, in which case only a thin scaffold of approximately 4 mm can be prepared. Gradients have been generated directly by salt leaching through pore distribution or pore size or indirectly by surface modification of the porous scaffold. Wu et al. developed a poly(L-lactic acid) (PLLA) scaffold by NaCl particle leaching.[61] The scaffold was placed vertically in a beaker and then aminolyzed along a gradient by wetting it at a controlled speed from bottom to top with a 1,6-hexanediamine–propanol solution. Gelatin was then immobilized by the amino groups via a glutaraldehyde coupling agent to form a gelatin gradient. Another example is a study by Orsi et al., who built gene-activated PEG scaffolds with two different pore size gradients using a gelatin particle template followed by DNA complex adsorption after gel photopolymerization and gelatin leaching.[62] One scaffold type showed a stepwise pore size gradient with 75–150 μm and 300–500 μm pore areas; the second type showed a continuous pore size gradient from 75 to 300 μm. The scaffolds were then loaded with NIH3T3 mouse embryo fibroblasts, and the culture was continued for 16 days. The results

showed that the cells did not penetrate the scaffold at the smallest pore size area, they slowly penetrated the scaffold in the 75–150 μm pore area, and they totally colonized the scaffold in the 150–300 μm and 300–500 μm pore areas. These studies demonstrated the importance of the pore sizes of gradient biomaterials.

3.3.2 Gas Foaming

In gas foaming, a polymer phase is saturated with a gas such as carbon dioxide at high pressure (800 psi). When the pressure inside the chamber is quickly released, gas bubbles are generated and grow in the polymer, a process called "foaming." Upon the completion of the foaming process, the polymeric scaffold turns into a 3D porous structure with an expanded polymeric volume and decreased polymeric density. The amount of dissolved carbon dioxide in the polymer solution determines the porosity and porous structure of the scaffolds. This process produces a sponge-like structure. A technical variation is the use of a chemical reaction with a gas foaming/blowing agent, such as ammonium or sodium bicarbonate, rather than a gas flow.[63] This technique allows for the pore size to range from 100 to 500 μm in the polymer and produces a good porosity, ranging from 60% to 97%, but has low pore interconnectivity and incomplete pore opening because of the formation of a closed external skin during the process.[55,64] To overcome these problems, gas foaming is often combined with microparticulate, salt leaching, or continuous templating techniques.[65–67] Researchers have shown that the microparticle–polymer ratio and particle size control the foam porosity and pore size. By combining gas foaming and sodium chloride microparticulate templating, Salerno et al. showed that increasing the sodium chloride concentration in the PCL polymer matrix from 30 to 80 wt% decreases the pore size in the foam from 71 to 10 μm.[68] These authors used this approach to control pore size and porosity through porogen salt concentration to build a graded scaffold by loading a PCL polymer phase with a sodium chloride concentration gradient from 30 to 60 wt%. They obtained a scaffold with a spatial porosity gradient decreasing from 91% to 83% and a pore size gradient decreasing from 71 to 24 μm. Different fillers, such as hydroxyapatite, β-tricalcium phosphate (β-TCP), carbon fibers, or glass fibers, can also be added to the polymer matrix to change the mechanical properties or bioactivity of the foams.[69] For example, Buhler et al. developed polylactic acid (PLA)-reinforced glass fiber composite graded scaffolds with a volume fiber gradient increasing from 0% in the middle of the scaffold to 10% at the borders and a porosity gradient decreasing from 85% in the center to 65% in the outer zones with an improved flexural modulus.[70] Numerous reports have demonstrated the efficacy of tissue scaffolds prepared by the gas-foaming method.

3.3.3 Phase Separation

In phase separation, a homogenous polymer solution demixes into a polymer-lean phase and a polymer-rich phase due to the addition of an immiscible solvent or to a decrease of the temperature below the solvent melting point. Subsequent freeze-drying of the liquid–liquid phase results in solvent removal and produces

microporous structures. Typically, this technique allows for the formation of micropores (1–10 μm), but it can also be used to generate macropores and to obtain a scaffold with a uniform pore size distribution and good interconnectivity and porosity (>90%).[71] Phase separation can easily be combined with other fabrication technologies (e.g., particulate leaching) to design 3D structures with controlled pore morphology. Although this technique has been effectively used by itself or in combination with other techniques to build tissue scaffolds, very few papers discuss constructing a gradient with phase separation. By combining phase separation and freeze-drying, Van Vlierberghe et al. built a gelatin hydrogel with a pore size gradient decreasing from 330 to 20 μm diameter and porosity gradients decreasing from 82% to 61% porosity.[72] These experimental data and others suggest the potential for the development of tissue engineering scaffolds using phase separation techniques.

3.3.4 Emulsification

In emulsification, a polymer is dissolved in an organic solvent followed by water addition, and the two phases are stirred to obtain an emulsion. The emulsion is then cast and quickly frozen by immersion into nitrogen liquid followed by freeze-drying to remove the dispersed water and solvent, giving to the scaffold a porous structure. Microgels can be constructed by using emulsification in another way. The purpose here is not to generate pores by removing aqueous droplets from a matrix but rather to generate microgel beads by removing the matrix or organic phase after cross-linking.[73,74] However, freeze-drying or lyophilization by itself is a commonly used technique for the fabrication of porous scaffolds, notably collagen sponges, by applying a temperature gradient during the freezing process. This type of scaffolding system has been used in tissue engineering. For example, Harley et al. designed a tubular scaffold with a radial pore size gradient by spinning the polymer solution during the freeze-drying process for a peripheral nerve regeneration application.[75] Near the lumen, the mean pore size was approximately 20 μm, allowing cell penetration from the lumen, whereas near the outer scaffold surface, the mean pore size was 5 μm, impairing cell penetration from the outside. Oh et al. built a PCL scaffold with a pore size gradient by centrifugation of fibril-like PCL in a cylindrical mold and gradually increasing the spinning speed.[15] This processing step was followed by a freeze-drying step and then by a heat fibril-bonding treatment. From top to bottom, the scaffolds obtained by this method had a porosity gradient decreasing from 94% to 80% and a pore size gradient decreasing from 405 to 88 μm. The scaffold's mechanical strength decreases with increasing porosity along the gradient axis. When this scaffold was loaded with a mixture of chondrocytes, osteoblasts, and fibroblast cells and cultured for more than 14 days, chondrocytes and osteoblasts grew well on the larger pore size regions (310–405 μm), whereas fibroblasts preferred to grow in the smaller pore size region (186–200 μm). The efficacy of this type of porous scaffold has also been demonstrated *in vivo*. The scaffolds were implanted without cells into rabbit skull defects, and new bone growth was noted in the region with a pore size of 290–405 μm. Therefore, emulsification-derived scaffolds also play an important role in tissue engineering.

3.3.5 Solid Free-Form Technology

Solid free-form technology (SFF) fabrication encompasses several techniques to manufacture solid structures by delivering energy or materials to specific points in space to produce the structure.[76] Some of these techniques include electron beam melting (EBM), fused deposition modeling (FDM), stereolithography (SLA), laminated object modeling, selective laser sintering (SLS), and 3D printing (3DP). For example, Roy et al. fabricated PLGA scaffolds containing 20 wt% β-tricalcium phosphate with a porosity gradient from 80% to 88% and with pore sizes ranging from 125 to 150 μm by 3DP.[77] Scaffolds implanted into 8 mm-diameter defects in rabbit calvaria showed a new bone density gradient matching the porosity gradient at 8 weeks after surgery. Kalita et al. built TCP–polypropylene (PP) composite scaffolds with a porosity gradient via FDM. Scaffold design was conducted on a computer using CAD software, and the TCP–PP filament was then weaved with different mesh sizes to obtain areas with different pore sizes.[78] In the same context, Lian et al. presented a computer model that reciprocally converts a 3D structure into two-dimensional (2D) stacking concentric patterns.[79] They then used this software to construct an epoxy resin mold via stereolithography and casted calcium phosphate cement (CPC) scaffolds. The software allows for the production of scaffolds with porosity gradients. Thus, SFF is a technique that can be used to fabricate tissue scaffolds with accurate designs or structures that match to specific tissue or organ defects.

3.3.6 Photolithography

Photolithography is a microfabrication technique that allows for the formation of distinct patterns with the desired geometry onto a biomaterial substrate that is suitable for cell studies. During this process, a photoresist polymer undergoes selective photopolymerization caused by selective UV irradiation through a photomask with the desired pattern geometry. Some of the early studies in the use of microfabricated structures and cells were conducted using this approach. For example, in the 1980s, Kleinfield et al. cultured neurons onto photolithographically patterned SAMs.[80] In general, although the pattern resolution obtained by this method is a few micrometers, a high resolution below 100 nm can be achieved using advanced techniques and materials.[81] This technique has many variations and potential applications, but it cannot be used to pattern molecules or biological materials that are UV sensitive. Surfaces patterned by photolithography can be used directly or as templates to generate other patterned surfaces. These surfaces then allow for precise cell manipulation and localization, which facilitates control of cell–cell and cell–material interactions. Although photolithography is basically a 2D process, it is possible to generate 3D structures in a photoresist with gradients in height and/or roughness using gray-scale technology.[82] This technique locally modulates UV exposure doses through a photomask, which has several gray levels, in contrast to conventional binary photomasks that are only black or translucent. Interesting work by Chen et al. showed that a gray photomask can be replaced by microfluidic channel patterns filled with liquids of different color levels and that a

gradient structure can be generated using liquids with decreasing opacity.[83] Thus, Wang et al. used a gray mask technology to generate a protein concentration gradient on an aminated glass coverslip.[84] Using a conventional photomask, Li et al. built a laminin density gradient on a poly(ethylene terephthalate) (PET) substrate by a two-step UV irradiation method.[85] These authors first generated peroxides on the PET surface with a first UV irradiation step and then grafted poly(acrylic acid) (PAA) onto the PET surface with a second UV irradiation step. This process was completed by covalently coupling the amino terminal groups of laminin proteins to the carboxyl groups of PAA. The gradient was generated by moving the substrate below the UV at a controlled speed during the first 3 min of the first UV irradiation, with the result that different areas on the substrate received different amounts of UV and therefore had different amounts of peroxides. When the coverslips were loaded with pheochromocytoma PC12 cells and cultured for 2 days, a cell density gradient matching the laminin density gradient was observed. Toh et al. built a single-density gradient of biotinylated lectin concanavalin A (ConA–biotin) and a double-density gradient of polysaccharide mannan and glycoprotein P-selectin on benzophenone (BP)-coated glass coverslips.[86] The process involved BP-diradical generation via the UV exposure of the substrate through a photomask with a simultaneous flow of biomolecules. BP diradicals then formed covalent bonds with proximal biomolecules. A ConA–biotin gradient was generated by a shutter with a controlled closing speed during UV irradiation, and the double gradients were generated by a controlled rotating shutter. When the resulting materials were loaded with promyelocyte HL-60 cells and cultured for 2 h, a cell density gradient was observed following the P-selectin gradient. When cells in suspension were flowed perpendicularly to the P-selectin gradient, a cell rolling velocity gradient matching the P-selectin gradient was observed. Photolithography can also be used to photopolymerize hydrogels,[87] which could provide a cross-linking density gradient[88] or a patterned gradient with different molecules such as RGD or particles. Based on numerous research studies, photolithography plays an important role in designing scaffold substrates suitable for basic cell studies.

3.3.7 Microfluidics

Microfluidics allows for the patterning of 3D structures suitable for controlling cellular functions. This patterning technique is closely related to microcontact printing. Instead of stamping a polydimethylsiloxane (PDMS) mold with a relief pattern of a master, a microfluidic network is stamped onto a substrate. In this method, the microchannels are used to deliver fluids to selected areas of a substrate, and the substrate is exposed to the flow, resulting in the patterning of the material. This method is frequently used to pattern multiple components on a single substrate and allows for the directed delivery of cells and soluble factors onto the substrate, making it important for applications in cell biology, drug screening, and tissue engineering. Unlike conventional *in vitro* cell culture methods, microfluidics can produce miniature and complex structures mimicking the *in vivo* cellular environment, which is one of the merits of this technique.

From the 1970s to 1990s, microfluidic devices were mainly constructed from silicon and glass substrates using technologies such as photolithography and etching. In the late 1990s, the introduction of soft lithography using polymer materials, in which channels can be molded or embossed rather than etched, allowed for the easier and cheaper fabrication of microfluidic devices. The most widely used polymer to build microfluidic devices for biological applications is PDMS[89–92] because of its material properties, including its biocompatibility, gas permeability, optical transparence down to 280 nm, and ability to replicate microscale features with high fidelity by replica molding. Moreover, PDMS-based soft lithography allows for rapid prototyping. One way to build a gradient with microfluidics is to fill an empty microchannel with capillary force. Density gradients of biomolecules such as proteins can be created in PDMS microchannels because of the adsorption of the biomolecules to the hydrophobic PDMS and because the microfluidic channels have a large surface-area-to-volume ratio, leading to the depletion of the biomolecule from the solution along the channel.[93] The channel outgas technique (COT) involves filling a PDMS microchannel through the inlet reservoir with a biomolecule solution, closing the outlet with a cover glass, and placing the device under vacuum before restoring atmospheric pressure.[94] This technique can produce a gradient from a few hundred micrometers to 1 cm. Another way to build a gradient with microfluidics is to use the diffusion between two liquids. A microchannel is prefilled with solution A, and then solution B is introduced and diffused into the channel.[95] A diffusion gradient with a parabolic shape moves along the channel with the forward flow. If an additional backward flow is generated from the inlet by evaporation, the parabolic profile is flattened, the lateral concentration distribution becomes uniform, and the concentration gradient stabilizes and elongates.[96] This technique can be used to create a centimeter-long gradient. If this backward flow continues, an inverted parabolic profile is formed by the concentration distribution, and the gradient moves backward toward the inlet. To stop this gradient displacement, the backward flow must be prevented by avoiding evaporation. This is done by sealing the inlet, for example by using oil, or by placing the device in a wet atmosphere. He et al. applied this diffusion strategy to build 1–5 cm-long poly(ethylene glycol)–diacrylate (PEG–DA) concentration gradient hydrogels.[39] After freeze-drying, the hydrogel presents a porosity gradient and a decreasing thickness toward low PEG–DA concentrations. Hydrogels were also prepared with the cell-adhesive ligand Arg-Gly-Asp-Ser (RGDS) in a gradient or at a constant concentration. Culturing human umbilical vein endothelial cells (HUVECs) on these hydrogels led to a cell density gradient and a cell morphology gradient (from round shape to well spread) following the RGDS concentration gradient or the PEG–DA concentration gradient when RGDS was constant. A programmed syringe pump can be used to induce repetitive forward and backward flows to lengthen the gradients. For example, Du et al. used alternate forward and backward flows with 30 s intervals between each sequence to allow for lateral mixing by diffusion.[97] Whereas convection stretches the fluid along the channel axis, diffusion acts laterally and tends to suppress hydrodynamic stretching. The use of high-speed (on the order of millimeters per second) flow improves the hydrodynamic stretching and allows for the generation of a long-range gradient of

molecules, microbeads, or cells. Du et al. used this dispersion-based technique to build an HA-gelatin composite hydrogel with a 2–3 cm cross-gradient of hyaluronic acid (HA), which is a cell repellent, and gelatin, which is a bioactive material. SMC cells were cultured for 24 h to study the effect of the cross-gradient, and the cell density gradient followed the gelatin concentration gradient.

Another method of generating gradients is to use laminar flows. Indeed, at a low Reynolds number (<1), no mixing by convection occurs between two adjacent flows, but diffusion is possible. Devices using this technique, such as the T-sensor,[98] involve several separated fluid streams that merge adjacently into a single microchannel. Gradients are generated perpendicular to the flow direction. In 2000, Jeon et al. demonstrated a method for the generation of gradients in chemical composition and surface topography using a microfluidic mixer. This system, built in PDMS, is based on a Christmas tree–shaped microfluidic network, which, from top to bottom, repeatedly splits the streams at the nodes, combines them with neighboring streams, and allows them to mix by diffusion in the serpentine channels.[99] At the end of the network, all streams carrying different concentrations of molecules of interest combine in a broad channel, generating a concentration gradient perpendicular to the flow direction. Using different fluids, a variety of gradients can be generated with a resolution of several microns to several hundred microns. Gradients with different shapes (symmetric or asymmetric), types (smooth, step, multipeaked),[100] and natures (static or dynamic) can also be obtained based on this technique. Using this technique, Burdick et al. built a PEG hydrogel with a gradient of adhesive ligands (RGDS).[30] When HUVECs were cultured on this hydrogel for 3 h, a cell density gradient was observed, matching the RGDS gradient with better spreading toward high RGDS concentrations. These data indicate that microfluidics is a useful technique for building systems that facilitate the studies of cell behavior required for the development of tissue engineering.

3.3.8 Microcontact Printing

Microcontact printing (μCP) is a well-known technique that allows for the transfer of patterns onto biomaterial substrates with high spatial resolution suitable for cell studies. This microfabrication technique is one of the best-known techniques in bioengineering because of its versatility and simplicity for patterning biomaterials without using any expensive equipment. This technique can also be used to pattern a nonplanar surface with a 3D structure when conventional photolithographic techniques would not be feasible. μCP was introduced by the Whitesides group in the early 1990s to replicate patterns generated by photolithography. Initially, this method used the spontaneous adsorption of alkylthiols to form SAMs on gold, which then resist gold etching with alkaline cyanide.[101] μCP was then extended to alkylsiloxanes on silicon oxide, resulting in numerous biological and biotechnological applications.[102,103] Subsequently, other molecular inks[104,105] and other substrates were used, giving rise to numerous variations of μCP.[106] In microcontact printing, a stamp made of a soft polymer, such as polydimethylsiloxane, is soaked in a molecular "ink" that is imprinted on the surface of a substrate.[107,108] The resolution

of the technique is on the order of a few micrometers.[109] However, optimization of the stamp building technique allows for a resolution under 100 nm.[110] Although the μCP technique is simple to use and has several merits, it also has a few drawbacks. For example, stamp swelling during the inking process can result in larger imprinted patterns or resolution problems caused by overdiffusion of the ink; in addition, stamp deformation, such as pairing, buckling, or roof collapse, during contact with the substrate surface results in distorted patterns. μCP has been widely used to modify surfaces, and gradients were built by applying different pressures on the stamp, varying the contact time between the stamp and the substrate, using a nonplanar stamp, gradually soaking the stamp in ink, or gradually depositing the ink on the substrate.[111] Von Philipsborn et al. proposed a protocol to print discontinuous gradients of axon-guidance proteins by a lift-off method or by a casting method.[112] After overnight culture with embryonic chick retinal ganglion cell axons, protein patterns were analyzed for their interactions with axons by fluorescent labeling. Thus, μCP is an important technique that can be used to fabricate bioengineered systems and devices.

3.3.9 Electrospinning

Electrospinning is an easy and versatile technique based on the ejection of a polymeric jet from the tip of an electrically charged syringe, the spinneret, followed by its collection onto a counter electrode, resulting in the formation of fibers with sizes usually ranging from 10 nm to a few micrometers. By manipulating the electrospinning process, the thickness and orientation (aligned or random) of nanofibers can be controlled to match the structure of the targeted tissue.[113] Electrospun fibers have adequate mechanical properties, high porosity, and a large surface-to-volume ratio, which are beneficial properties for interactions with cells and for tissue engineering applications.[114,115] Thus, nanofiber scaffolds have been widely investigated for ligament,[116] meniscus,[117] and bone tissue engineering.[118,119] Nanofiber surfaces can also be modified by bioactive molecules to increase their cellular compatibility.[120,121] One approach to generating a gradient is based on the surface modification of the ejected fibers after electrospinning. Shi et al. incorporated a fibronectin concentration gradient in a polymethylglutarimide (PMGI) electrospun mesh scaffold by wetting the scaffold at a controlled speed from bottom to top with fibronectin solution in a vertical PDMS microchamber.[122,123] Loaded with NIH3T3 cells and cultured for 24 h, the scaffold showed a cell density gradient decreasing from 1400 cells/mm^2 (at the bottom of the scaffold) to 100 cells/mm^2 (at the top of the scaffold), following the fibronectin concentration gradient. To mimic the tendon–bone interface, Li et al. coated the electrospun nanofibers of PLGA and PCL mats with a calcium phosphate mineralization gradient by wetting the scaffolds at a controlled speed from bottom to top with 10-fold concentrated simulated body fluid (10 SBF) solution in a glass.[7] The mineralization gradient decreased from 37.8% (at the scaffold bottom) to 0.7% (at the scaffold top) in calcium phosphate and from 33.9% to 0.8% for the PLGA and PCL scaffolds, respectively. When the PLGA scaffold is subjected to uniaxial tensile deformation, the Young's modulus follows

the mineralization gradient and decreases with the decrease in mineralization. This stiffness gradient induced by the mineralization gradient mimics the stiffness distribution at the tendon–bone interface. In contrast, when the PCL scaffold was loaded with mouse preosteoblast MC3T3-E1 cells and cultured for 3 days, it showed a cell density gradient decreasing from 435 cells/mm^2 (at the bottom of the scaffold) to 115 cells/mm^2 (at the top of the scaffold) following the mineralization gradient, and the cells were oriented toward the higher mineralization area. The same group also fabricated a PLGA electrospun nanofiber scaffold with a random fiber orientation area and an aligned fiber orientation area combined with a calcium phosphate mineralization gradient decreasing from a random to an aligned fiber orientation to mimic both the composition and structure seen at the bone–tendon interface.[124] Recently, coupling microfluidics with the laminar flow of polymers to electrospray and electrospinning techniques, Lahann et al. have obtained multicompartmental spherical particles and aligned biodegradable PLGA multicompartmental microfibers with narrow polydispersity.[125–127] Because microfluidic systems can generate gradients, it is possible to couple a gradient microfluidic generator to the electrospinning setup, which would allow for the direct electrospinning of graded fibers. The experimental data obtained from these and other studies demonstrate that electrospinning can be used to fabricate scaffolds with gradients in physical and chemical properties mimicking the natural ECM at the interface zones.

3.3.10 Nanoimprint Lithography

Nanoimprint lithography (NIL) is a cost-effective and high-throughput technique for fabricating nanopatterns. NIL does not require any expensive instrumentation or sophisticated clean-room facilities, which are required for conventional lithographic techniques such as photolithography; thus, NIL is more suitable for biological applications. Moreover, the repartition of chemical compounds on the structured surface can be controlled. This technique can be applied to create 2D or 3D nanotopographical patterns of different geometry and can be used on a wide range of biomaterial substrates suitable for cell engineering. In NIL,[128] a thermoplastic or UV-curing polymer layer is imprinted by a mold and cured by heat (hot embossing or thermal-NIL) or by UV irradiation (UV-NIL) at the same time. In the latter case, the mold is usually in quartz so that it is permeable to UV. After cooling down or UV curing, the stamp is removed from the imprinted polymer, which contains the reversed stamp topography. This technique allows for a resolution of a few nanometers.[129] Limited data are available on generating gradients by nanoimprinting methods for biological applications. For a DNA stretching application, Cao et al. used nanoimprint lithography to build a structure with a size gradient from the micrometer to nanometer scale to overcome the difficulties of introducing long-genomic DNA molecules into nanometer-scale channels.[130] In two short preliminary reports, Sun et al. built patterns of parallel line and space gratings on polystyrene or dimethylacrylate surfaces with height gradients from 0 to 360 nm using nanoimprinting technology.[131,132] When murine preosteoblast MC3T3-E1 cells were loaded onto these surfaces and cultured for

2 days, a cell alignment and elongation gradient was observed that decreased with the height pattern.

3.3.11 Inkjet Printing

Inkjet printing is a noncontact reprographic method that translates numerical data from a computer into a pattern on a substrate using ink drops.[133] This technology is widely used in the electronics industry to print integrated circuits.[134] In the late 1990s, inkjet printing was adapted to biological applications with the printing of SAMs, DNA arrays, and other proteins.[135,136] Notably, this technology was used to pattern cells by printing ECM molecules.[137] Recently, it has become possible to directly print living cells, opening up an avenue to applications in tissue engineering and organ printing.[138,139] Indeed, it is possible to build a 3D structure by printing superimposed cell layers. Recent works have reported the printing of 3D hydrogels and hydrogels with cells.[140] Inkjet printing allows for high-precision cell positioning with a resolution of approximately 100 μm with a bio-ink and fast prototyping and manufacturing.[141] In color printers, inks can be replaced by different biological components, such as proteins, peptides, growth factors, polymers, drugs, or different cell lines, and all components can be printed simultaneously onto a culture dish, culture sheet, 3D scaffold, gel, or liquid. Because gradients can be easily designed on a computer by continuously fading a color, cell gradient patterns can be built by printing a collagen solution with a decrease in the spatial density of the bio-ink droplets.[137] Another strategy for printing gradients consists of overprinting, in which several bio-ink depositions are performed on the same spot. A density gradient can be obtained by controlling the number of overprints in a spatial repartition. Thus, Campbell et al. printed a fibroblast growth factor-2 (FGF-2) gradient on fibrin film using an overprinting strategy.[142] After 4 days of culturing the film with human MG-63 osteosarcoma cells, the team observed a cell density gradient following the hormonal gradient. Because cells or bacteria can be directly printed, Xu et al. printed bacterial density gradients of *Escherichia coli* on an agarose-coated coverslip by using an *E. coli* suspension as a bio-ink.[143] Ilkhanizadeh et al. printed different protein gradients on Hydrogel-coated slides from Perkin Elmer.[47] These authors showed that a printed gradient of Cy5-conjugated transferrin exhibited good stability in culture medium at 37°C over 22 h, which is enough time to induce a cellular response. In another example, Ilkhanizadeh et al. printed a CNTF gradient on a hydrogel. Because CNTF induced the differentiation of NSCs into astrocytes, which express GFAP, these authors observed a GFAP-positive cell density gradient decreasing from 14% to 6%, reflecting the printed CNTF gradient. NIL is an emerging technique for the development of scaffold systems with various biological applications.

3.3.12 Gradient Makers

The development of gradient makers dates from the 1960s. One interesting idea was presented by Alberto, who adapted a conventional 30 ml syringe to an exponential

gradient maker that can be used to make a gradient material.[144] The syringe outlet is plugged to a tube that is clamped. The syringe body is half filled with solution A with no concentration of the molecule of interest and a stir bar (chamber 1). The syringe plunger is modified by notching the up and down borders of the rubber sealing tip. The notched plunger is pushed into the syringe until no air space remains. Then the upper part of the syringe body is filled with solution B with a high concentration of the molecule of interest (chamber 2). When the outlet is unclamped, solution A begins to flow out, and solution B begins to flow into chamber 1 via the notched rubber and mix with solution A. Current gradient makers[144] are similar, consisting of two chambers, A and B, connected at the bottom by a pipe with a valve. Chamber B is also connected at the bottom to a peristaltic pump by an outlet pipe. Chamber A contains a solution with a high concentration of the molecule of interest, and chamber B contains a solution without or with a low concentration of the molecule of interest and a stir bar. When the valve is opened and the pump is started, the solution from chamber A is drawn into the pipe and mixed completely with the chamber B solution before it is delivered by the outlet pipe. A gradient is formed because the solution from chamber A is mixed with a decreasing volume of solution from chamber B. These types of gradient makers have been proposed by companies such as CBS Scientific, Hoefer, and GE Healthcare. Many studies have used a gradient maker to fabricate gradient biomaterials. For example, Chatterjee et al. used a gradient maker to fabricate a PEG hydrogel with a gradient of PEG concentrations ranging from 5% to 20%, resulting in a gradient of compressive modulus from 10 to 300 kPa. The encapsulation of MC3T3-E1 cells showed that the material property induced a screening of cell differentiation and showed a gradient of mineralization, which revealed that osteoblasts differentiate in the hydrogel region with a modulus of 225 kPa or greater.[145] These data indicate that gradient makers have great potential for designing gradient biomaterials suitable for tissue engineering.

3.4 CONCLUSIONS

Gradient biomaterials are new arrivals to the field of tissue engineering, and their introduction has led to the development of ITE. In contrast to conventional homotypic tissue engineering, ITE requires specially designed biomaterials that can mimic the structure and function of native heterotypic interface tissues, which contain several gradient features. Therefore, the development of biomaterials with gradients in mechanical properties, composition, structure, or incorporated biomolecules is essential. Micro- and nanotechnologies allow for the fabrication of such gradient biomaterials and can be used to create new, advanced gradient biomaterials for ITE applications. This chapter discussed some widely used techniques for the fabrication of gradient biomaterials and considered their merits and shortcomings as well as how the various gradient biomaterials can be used in basic cell studies and tissue engineering. Future developments of gradient biomaterials for ITE applications will include the design of new engineered surfaces and drug-releasing scaffolds, modified with several bioactive molecules, such as growth factors,

enzymes, ECM proteins, and DNA, to facilitate the tissue regeneration process by mimicking the ECM environment. In this regard, the inclusion within the scaffolds of the temporal control of the activity of these bioactive molecules to complement the spatial gradients would be an advantage. Indeed, the native ECM contains a plethora of physical and chemical cues that often exist in gradients and actively and temporally induce cellular responses such as migration and differentiation. Other points to be addressed for the fabrication of biomimetic scaffolds will be to localize the distribution of the physical and chemical properties within the scaffold and to favor the cooperation of heterotypic cells in the scaffold as well as with the surrounding host environment at the insertion sites. This interfacial tissue regeneration should result in the formation of a tissue with gradient properties in terms of cell type and ECM components. Thus, gradient biomaterials hold great promise for the field of ITE.

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4

MICROENGINEERED POLYMER- AND CERAMIC-BASED BIOMATERIAL SCAFFOLDS: A TOPICAL REVIEW ON DESIGN, PROCESSING, AND BIOCOMPATIBILITY PROPERTIES

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4.1 INTRODUCTION

Biomaterials are a class of engineering materials that can be used in tissue replacements, reconstructions, and regeneration without any long-term adverse effect. The development of biomaterials and manufacturing techniques broadened the diversity of applications for various biocompatible materials. A synthetic material or processed natural material is engineered to treat or replace any component or function of a biological organism while in continuous or intermittent contact with biological cells or tissues. Any natural or synthetic material complying with this definition is broadly classified as a biomaterial. In a nutshell, "A biomaterial is a substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine".[1] However, in this regard, biocompatibility is of paramount

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importance because debatable safety of the biomaterial may render its application questionable. As defined by David Williams, "Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy."[2]

Depending on the host response and biocompatibility, biomaterials can be typically classified under three categories[3]:

1. *Bioinert or biotolerant* materials, which, although biocompatible, fail to induce interfacial biological bond between natural bone and synthetic implant.
2. *Bioactive* materials, which are biocompatible and can easily attach with the body tissues, forming chemical and biological bond at an early stage in the postimplantation period.
3. *Bioresorbable* materials, which are gradually resorbed and are replaced by new tissues *in vivo*.

As mentioned earlier, a biomaterial must be obligatorily biocompatible (i.e., it must not educe unresolved inflammatory response or demonstrate immunogenicity or cytotoxicity). Additionally, the biomaterial scaffold should be mechanically strong enough, so as not to collapse during handling and the postimplant activities of the patient. Also, tissue scaffolds must be easily sterilizable to avert chances of infection.[4] A further requirement for a scaffold, particularly in hard tissue engineering, should have tailorable interconnected porosity to direct the cells to grow into desired physical form and boost vascularization of the ingrown tissue. In fact, a typical porosity of 90% and a minimum pore diameter of 100 μm are highly desired for cell penetration and proper vascularization of the ingrown tissue.[5–7] Furthermore, scalability, near-net-shape fabrications are highly desirable for cost-effective large-scale production of scaffold materials.

For an immensely complicated system such as the human body, amalgamating the biological and structural properties of a tissue into a biomaterial to engineer germane scaffolds presents a mammoth challenging task. It must be remembered that biomaterials are not subservient vehicles for introduction of cells into the diseased spot whatsoever, but they must be equally proficient in nurturing the endogenous progenitor cells functionally.

The use of materials as part of surgical implant is not new. More specifically, biomaterials in the form of sutures, bone plates, joint replacements, ligaments, and vascular grafts and medical devices such as pacemakers, biosensors, artificial hearts, and blood tubes are widely used to replace or restore the function of traumatized or degenerated tissues or organs, assist in healing, improve function, correct abnormalities, and thus improve the quality of life of the patients. The substitution of bone parts in the body has been done since the pre-Christian era. By the middle of 19th century, medical science attempted to repair body parts with synthetic materials.

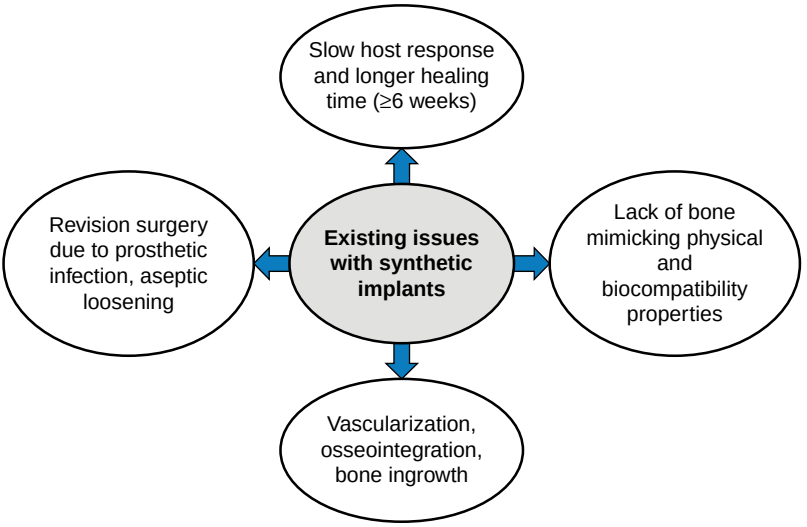

FIGURE 4.1 Schematic summarizing existing issues with synthetic implants. Adapted from Ref. [20], with permission from John Wiley and Sons.

In 1880, Gluck[8] used ivory prosthesis as implants in the body. In 1902, gold was used in capsule form interposing between the articular heads of an implant.[9] This was a big success, which leads to more study on chemically inert and stable materials. In 1972, Boutin started to study on ceramics such as alumina and zirconia, which did not have biological drawback and were considered everlasting.[10] But both of these ceramics were inert, so implantation was performed without cement anchorage to the tissue. This led to implants loosening very quickly. Such loosening leads to clinical failure, including fracture of the implant or the bone adjacent to the implant. Figure 4.1 summarizes the various existing issues with the synthetic implants such as host response, bone ingrowth, biocompatibility properties, and so on. To improve this unpromising outlook, biologically active or bioactive materials were developed, such as bioglass and hydroxyapatite (HA) by Hench[11] and Jarcho,[12] respectively.

Broadly, all biomaterials are being developed to attain a balance between the physical properties of the replaced tissues and the biochemical effects of the material on the tissue. A summary of the combination of aspects related to processing as well as biological properties to be considered while developing bone analogue materials are provided in Figure 4.2. Despite significant research on biomaterials,[13–19] it has been realized that synthetic materials cannot mimic the extremely complex structure of bone in all aspects and the important disadvantage of synthetic biomaterial is that they cannot repair themselves as living bone does.[20] In addition, the scaffolding offers the opportunity to introduce growth factors into the body. The highly porous materials that are used for scaffolding can be modified with biomolecules, which enhance the ability of the cells to migrate and grow. In this way, the scaffolding does not function simply as a physical structure but instead triggers the proliferation of

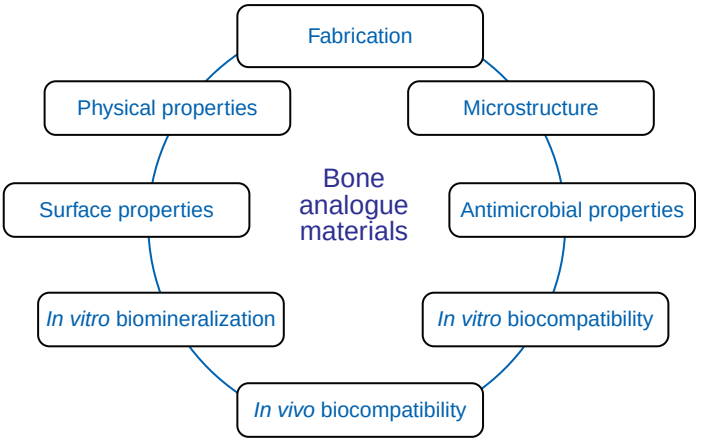

FIGURE 4.2 Schematic illustrating various approaches or issues to be critically considered while developing bone analogue materials. Adapted from Ref. [20], with permission from John Wiley and Sons.

cells and encourages the surroundings essential for tissue repair. Another approach of tissue engineering is to work with the body's tissues *in vitro*. In this approach, a small sample of cells is taken from the body, usually with a needle, and then the cells are grown in great number in a laboratory (Fig. 4.3).[21] They may still be grown over scaffolding to give them the necessary shape. These tissues can then be transplanted back into the body.

A summary of the combination of aspects related to fabrication as well as physical and biological properties of bone analogue materials is given in Figure 4.4. As far as the fabrication and microstructure are concerned, there is a wide range of processing techniques, which provide variation in microstructure. Among the physical properties, strength, modulus, and toughness are the important parameters. The physical properties are related to the microstructure as well the surface properties of the as-processed material. Surface properties include the surface roughness, porosity,

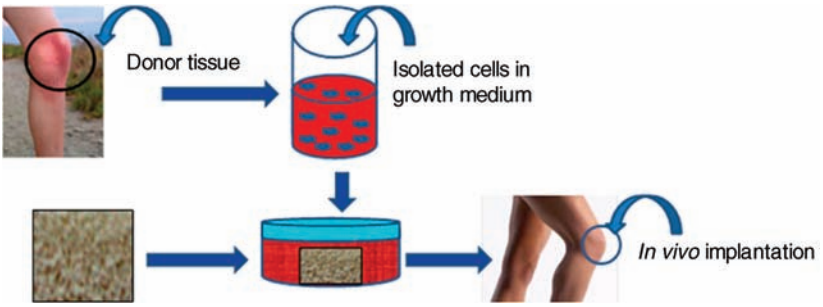

FIGURE 4.3 Schematic showing the tissue-engineering concept using a hypothetical example of implantation of scaffold for leg regeneration. Adapted from Ref. [20], with permission from John Wiley and Sons.

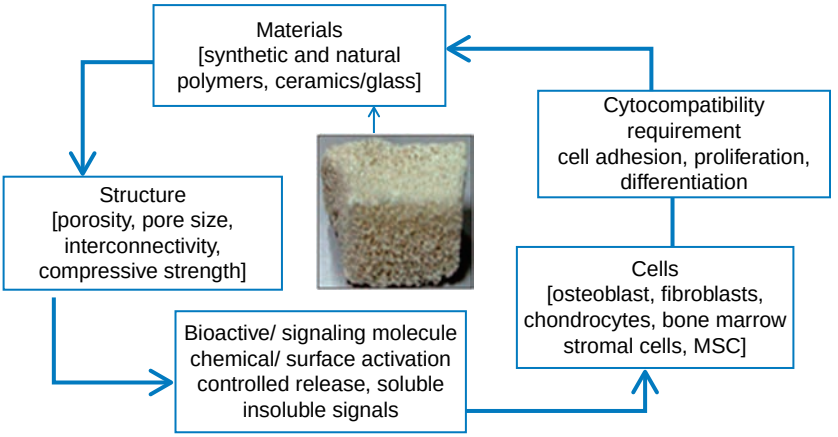

FIGURE 4.4 Property requirements for porous scaffolds for bone tissue engineering. MSC, mesenchymal stem cell. Idea adapted from Ref. 53.

charge, and wettability of the material. Antimicrobial properties of a material render resistance toward the bacterial adhesion and therefore the prosthetic infection. Among biological properties, cellular functionality and cell fate process are to be considered. *In vivo* osseointegration is important for hard tissue replacement applications. It is impossible to optimize the array of properties in a unique material composition. Therefore, a synergistic approach to combining various properties in designed composite materials is a possible solution.

Considering the options for various functional artificial biomaterials, the choice has to be made among metals, polymers, and ceramics. Each group exhibits some *a priori* advantages and drawbacks. Ceramics, for instance, are the most biocompatible materials and can be obtained with biostable, bioactive, or bioresorbable properties. They are well known for their good bioactivity, corrosion resistance, high compression strength, and high hardness. At the same time, they also have some drawbacks such as low fracture toughness and high stiffness. The elastic modulus of ceramics is at least an order of magnitude higher than those of hard tissues. Therefore, one of the major problems in orthopedic surgery is the significant difference between the stiffness of the bone and ceramic implants. As a result, the bone is insufficiently loaded compared with the implant; this phenomenon is known as "stress shielding" or stress protection. On the other hand, metals exhibit problems of corrosion and toxicity, but their mechanical strength and toughness are superior to those of ceramics. Polymers offer many possibilities depending on their chemical composition and structure (e.g., biodegradability degree, hydrophilic–hydrophobic ratio, toughness or flexibility), but very few have shown good bioactive properties to ensure implant osteointegration. Therefore, it is important to reach the best compromise possible, and it is quite usual to use two or more types of materials in the same implant. Polymer–ceramic or polymer–inorganic composites could be the alternative way to overcome many shortcomings, as mentioned earlier.

This chapter is structured into following sections. After the introductory section and general overview of biomaterials, the section on dense HA versus porous HA scaffold emphasizes the necessity of porosity for better *in vitro* and *in vivo* properties. The section on property requirements of porous scaffold describes the fundamental aspects of these essential requirements. After this, the design criteria and critical issues with porous scaffolds for bone tissue engineering are discussed. The next section provides an exculpation of porous scaffolds. Section 4.5 summarizes the requirement of porosity and future application. A detailed discussion follows on various fabrication processes for porous scaffolds, including a brief discussion on the advantages and disadvantages of typical processing routes in Section 4.6. Section 4.7 provides an overview of physicomechanical property evaluation of porous scaffold. The biological property evaluation in terms of *in vitro* and *in vivo* assay results reported by various researchers is summarized in Section 4.8. This chapter closes with the mention of some outstanding issues related to future research on porous scaffold.

4.2 DENSE HYDROXYAPATITE VERSUS POROUS HYDROXYAPATITE SCAFFOLD

The inorganic phase of our bones is apatite, more commonly known as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$).[22] Its structure has the special ability to accommodate several different ions in its three sublattices.[23,24] Bone apatites could be considered as basic calcium phosphates. To work for potential hard tissue replacement solutions, it is essential to know the bone regeneration process. Wolf's law dictates that the bone remodels itself as a function of forces acting on it, hence preserving its shape and density.[25] The mechanical loads of stress, compression, flex, and torsion in bones and the interstitial fluid contained in them generate stresses and deformations at the microscopical level, which in turn stimulate the bone cells.[26]

Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] is one of the most widely studied inorganic material and is well known for its biocompatibility, bioactivity, high osteoconductivity, and relatively high strength and modulus.[27,28] HA is the most important bioceramic materials for its unique bioactivity and stability. Unlike other calcium phosphates, HA does not break down under physiological conditions. In fact, it is thermodynamically stable at physiological pH and actively takes part in bone bonding, forming strong chemical bonds with surrounding bone. This property has been exploited for rapid bone repair after major trauma or surgery. Although its mechanical properties have been found to be unsuitable for load-bearing applications such as orthopedics, it is used as a coating on load-bearing implant materials such as titanium and titanium alloys or composites with other materials.

Porous HA ceramics have found enormous use in biomedical applications, including bone tissue regeneration, cell proliferation, and drug delivery. HA with controlled porosity is analogous to the natural ceramic in human bone. It is bioactive in the sense that interfacial bonds can develop between HA and the living tissues, leading to enhanced mechanical strength of the overall structure. However, the lower

TABLE 4.1 Pore Size Distribution for an Ideal Scaffold in Bone Tissue Engineering Applications

Pore Size	Biological Function
$<1\ \mu m$	Protein interaction; responsible for bioactivity
$1–20\ \mu m$	Cell attachment; their orientation of cellular growth (directionally)
$100–1000\ \mu m$	Cellular growth and bone ingrowth
$>1000\ \mu m$	Shape and functionality of implant

Idea adapted from Ref. [29].

mechanical strength of pure HA has hampered its use as a bone implant material because of conflicting requirements of porosity and strength.

Porous HA exhibits strong bonding to the bone; the pores provide a mechanical interlock leading to a firm fixation of the material. Bone tissue grows well through the pores, thus increasing strength of the HA implant *in vivo*. The ideal bone substitute material should form a secure bond with the tissues by encouraging new cells to grow and penetrate. New tissue and bone formation can easily take place on osteophilic and porous implant and also helps to prevent loosening and movement of the implant. When pore sizes exceed 100 μm, the bone grows through the channels of interconnected surface pores, thus maintaining the bone's vascularity and viability. The application of implant depends on the pore size, as summarized in Table 4.1.[29] Because porous HA is more resorbable and more osteoconductive than dense HA, there is an increasing interest in the development of synthetic porous HA bone replacement material for the filling of both load-bearing and nonload-bearing osseous defects. In terms of simulating the human bone structure, porous HA scaffold has a large surface area, which is beneficial for adhesion of biological cells and growth of new bone phase.

4.3 PROPERTY REQUIREMENT OF POROUS SCAFFOLD

Scaffold properties depend primarily on the nature of the biomaterial and the fabrication process. The scaffolds are based on various materials, such as metals, ceramics, glass, chemically synthesized polymers, natural polymers, and combinations of these materials to form composites. The properties and requirements for scaffolds in bone tissue engineering have been extensively reviewed; recent examples include aspects of degradation,[30–33] mechanical properties,[34–38] cytokine delivery,[39–43] and combinations of scaffolds and cells.[44,45]

Porosity is defined as the percentage of void space in a solid,[46] and it is a morphological property, independent of the material. Pores are necessary for bone tissue formation because they allow migration and proliferation of osteoblasts and mesenchymal cells as well as vascularization.[47] In addition, a porous surface improves mechanical interlocking between the implant biomaterial and the surrounding natural bone, providing greater mechanical stability at the critical interface.[48] The most common techniques used to create porosity in a biomaterial are salt

leaching, gas foaming, phase separation, freeze-drying, and sintering, depending on the scaffold material. The minimum pore size required to regenerate mineralized bone is generally considered to be ~100 μm according to the study by Hulbert et al., and this is on the basis of implantation experiments on calcium aluminate cylindrical pellets with 46% porosity in dog femurs.[49] Whereas large pores (100–150 and 150–200 μm) showed substantial bone ingrowth, smaller pores (75–100 μm) resulted in ingrowth of unmineralized osteoid tissue. Smaller pores (10–44 and 44–75 μm) were penetrated only by fibrous tissue. These results were correlated with normal haversian systems that reach an approximate diameter of 100–200 μm. In a different study, titanium plates with four different pore sizes (50, 75, 100, and 125 μm) were tested in rabbit femoral defects under nonload-bearing conditions.[50] Bone ingrowth was similar in all the pore sizes, suggesting that 100 μm may not be the critical pore size for nonload-bearing conditions.

Scaffold materials can be synthetic or biologic and degradable or nondegradable, depending on the intended use.[51] Various scaffolds can be categorized into different types in terms of their structural, chemical, and biological characteristics (e.g., ceramics, glasses, polymers). Naturally occurring polymers, synthetic biodegradable, and synthetic nonbiodegradable polymers are the main types of polymers used as biomaterials. It is known that the properties of polymers depend on the composition, structure, and arrangement of their constituent macromolecules.

Scaffolds for bone tissue engineering are subjected to many interlinked and often opposing biological and structural requirements. A major hurdle in the design of scaffolds is that most of the materials are either mechanically strong or bioinert, while degradable materials tend to be mechanically weak.[52] Hence, the fabrication of composites comprising biodegradable polymers and ceramics becomes a suitable option to fulfill the requirements of bioactivity, degradability, and mechanical competence. The desired features of a scaffold, such as interconnectivity, pore size and curvature, and surface roughness directly influence cellular responses, and they also control the degree of nutrient delivery, penetration depth of cells, and metabolic waste removal.[53] The design criteria and critical issues with porous scaffolds for bone tissue engineering are summarized in Figures 4.4 and 4.5.

It is important to meet some criteria while developing the porous scaffold to fulfill the requirements of bone tissue engineering (Figs. 4.5 and 4.6). The property requirements include (1) it must be biocompatible, which enables the cell growth, their attachment to surface, and proliferation; (2) the material should induce strong bone bonding, resulting in osteoconduction and osteoinduction; (3) the rate of new tissue formation and biodegradability should match with each other; (4) the mechanical strength of the scaffolds should be adequate enough to provide mechanical constancy in load-bearing sites before regeneration of new tissue; and (5) porous structure and pore size should be more than 100 μm for cell penetration, tissue ingrowth, and vascularization (see also Table 4.1).[29] As named, porosity is an important factor to allow cells to migrate via pores. The interconnected pores allow cells to migrate in multiple directions under *in vitro* and *in vivo* conditions.

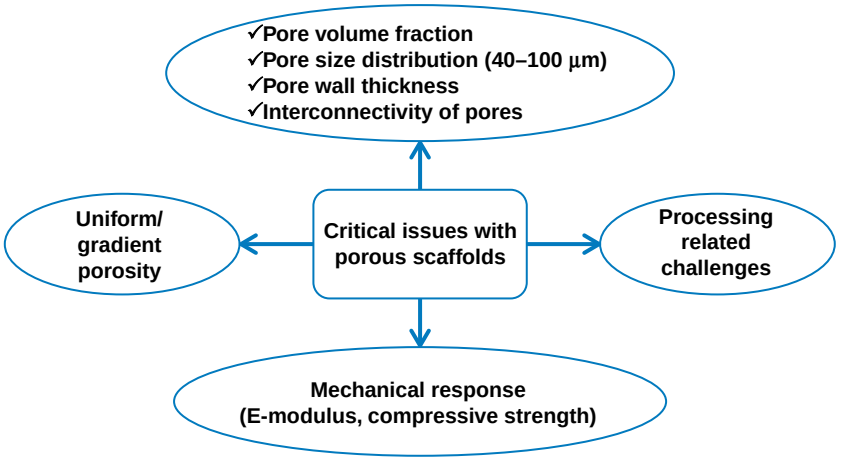

FIGURE 4.5 Critical issues with porous scaffold for bone tissue engineering.

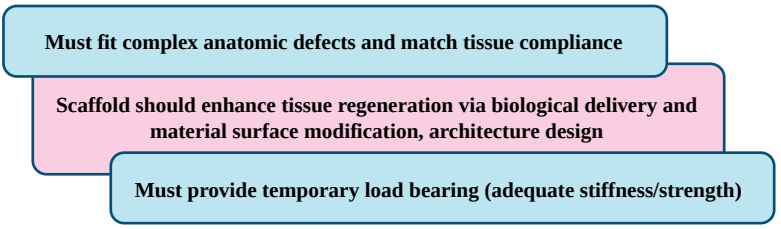

FIGURE 4.6 Essential requirements of porous scaffolds.

4.4 DESIGN CRITERIA AND CRITICAL ISSUES WITH POROUS SCAFFOLDS FOR BONE TISSUE ENGINEERING

4.4.1 Cytocompatibility

The most important characteristic feature is compatibility of the implant or porous scaffold with biological cells or tissue. The material should not only be cytocompatible but also foster cell attachment, differentiation, and proliferation. The cellular functionality on porous scaffolds is cell-type dependent, and normally, *in vitro* cell culture assay is performed using osteoblast-like cells or fibroblast-like cell proliferation and adhesion behavior. Also, cell viability in terms of metabolically active cells is measured using a number of assays such as MTT, LDH, and so on. Similarly, the cell differentiation behavior is investigated using an ALP or osteocalcin assay.

4.4.2 Osteoconductivity

According to Wilson-Hench,[54] osteoconduction is the process by which bone is directed so as to conform to a material's surface. However, Glantz[55] has pointed out that this way of looking at bone conduction is somewhat restricted because the original definition bears little or no relation to biomaterials. Osteoconductivity essentially indicates the lack of fibrous tissue encapsulation but also reflects on the possibility of formation of a strong bond between the scaffold and host bone.

4.4.3 Porous Structure

Pores are routinely created in scaffolds to promote three-dimensional (3D) tissue growth, nutrient diffusion, and vascularization. The size of pores must be large enough to allow the circumferential attachment of cells yet small enough to encourage migration and proliferation. The scaffold should have an interconnected porous structure with porosity of more than 90% and pore sizes between 100 and 500 μm, which would be helpful for cell penetration, tissue ingrowth, vascularization, and nutrient delivery.

4.4.4 Mechanical Properties

The mechanical strength of the scaffold should be sufficient to provide mechanical stability to constructs in load-bearing sites before synthesis of new extracellular matrix by cells. The scaffolds should have enough compressive strength, depending on the intended site of application.

4.4.5 Biodegradability

The composition of the material, combined with the porous structure of the scaffold, should lead to biodegradation *in vivo* at rates appropriate to tissue regeneration. Cell transplantation using biodegradable polymer scaffolds offers the possibility to create completely natural new tissue and replace organ function. Tissue-inducing biodegradable polymers can also be used to regenerate certain tissues without the need for *in vitro* cell culture. Also, the biodegradable polymers play an important role in organ regeneration as temporary substrates to transplanted cells, which allow cell attachment, growth, and retention of differentiated function.

4.4.6 Fabrication

The material should possess the ability to be fabricated into irregular shapes of scaffolds, which could match with the defects in bone of individual patient. The synthesis of the material and fabrication of the scaffold should be suitable for commercialization. The ease as well as reproducibility should be considered to select a processing route to fabricate porous scaffolds. The scaffolds should have good enough compressive strength, depending on the intended site of application.

A scaffold should provide an open porous network structure, allowing easier vascularization, which is important for the maintenance of penetrating cells from surrounding tissues and the development of new bone *in vivo*. The higher the macroporosity, the easier the vascularization of implant. The failure to develop an adequate vascular network means that only peripheral cells may survive or differentiate, supported by diffusion. Chang et al.[56] proposed that the degree of interconnectivity rather than the actual pore size has a greater influence on osteoconduction. Interconnectivity is a physical characteristic that aids in the delivery of nutrients and removal of metabolic waste products. Some studies have shown that bone normally forms in the outer 300 μm periphery of scaffolds and that this may be explained by the lack of nutrient delivery and waste removal.[57] When the pore size is too small, pore occlusion can occur by cells, preventing further cell penetration and bone formation.[58] Pore size distributions for an ideal scaffold in bone tissue engineering applications are summarized in Table 4.1. It is pertinent to note that much higher rate of mass transfer exists at the periphery of a scaffold and that these higher rate promote mineralization, further limiting the mass transfer of nutrients to the core of a scaffold.[59] It is essential that a scaffold possess a high degree of interconnectivity in conjunction with a suitable pore size to minimize pore occlusion.

4.5 AN EXCULPATION OF POROUS SCAFFOLDS

The concept behind nearly inert, microporous bioceramics is the ingrowths of tissue into pores on the surface or throughout the implant. The porosity is a critical factor for growth and integration of a tissue into the bioceramic implant. In particular, the open porosity, which is connected to the outside surface, is critical to the integration of tissue into the ceramic, especially if the bioceramic is inert. The increased interfacial area between the implant and the tissues results in an increased inertial resistance to movement of the device in the tissue. The interface is established by the living tissue in the pores. This method of attachment is often termed *biological fixation*. The limitation associated with porous implants is that for tissue to remain viable and healthy, it is necessary for the pores to be greater than 100–150 μm in diameter. The large interfacial area required for the porosity is because of the need to provide blood supply to the ingrown connective tissue. Vascular tissue does not appear in pores, which measure less than 100 μm. If micromovement occurs at the interface of a porous implant, tissue is damaged, the blood supply may be cut off, tissue dies, inflammation ensues, and the interfacial stability can be destroyed.

The potential advantage offered by a porous ceramic is the inertness combined with the mechanical stability of the highly convoluted interface when the bone grows into the pores of a ceramic. The mechanical requirements of prostheses, however, severely restrict the use of low strength porous ceramics to low-load or nonload-bearing applications. Studies show that when load bearing is not a primary requirement, nearly inert porous ceramics can provide a functional implant. Apart from biological aspects, the mechanical requirement should also be fulfilled by the engineered implant.

The necessity for porosity in bone regeneration has been shown by Kuboki et al.[60,61] using *in vivo* experiments in a rat model. Solid and porous particles of HA for bone morphogenic protein 2 (BMP-2) delivery were investigated simultaneously. Whereas no new bone formation was found on the solid particles, in the porous scaffolds, direct osteogenesis occurred. Further support comes from studies with porous-coated metallic implants compared with noncoated material. The treatment of titanium alloy implant surfaces with sintered titanium beads created a porous coating that enhanced the shear strength of the implants recovered from sheep tibia, but further coating with HA beads did not result in significant improvement. Titanium fiber-metal porous coatings (45% porosity and 350 μm average pore size) maximized bone ingrowth and increased the potential for stress-related bone resorption of femoral stems in a canine total hip arthroplasty model.[62] A similar result was observed for plasma spray-coated titanium implants with 56–60% porosity, although bone ingrowth was maximized for an open-pore titanium fiber mesh (60% porosity and 170 μm average pore size) coated with polyvinyl alcohol hydrogel.[63] D'Lima et al.[64] showed that surface roughness was more important for osseointegration of titanium implants in rabbit femurs because an acid-etched coating (highest surface roughness) showed a higher overall osseointegration compared with grit-blasted fiber mesh (average pore size, 400 μm) coatings. The coating of titanium alloy implants with a 50 μm layer of porous HA did not increase the percentage of osseointegrated surface in the mandibles of dogs, although bone extended into the micropores of HA, resulting in an osseous microinterlocking.[65] However, there was more bone opposing the coated implants in the maxillae, suggesting a beneficial effect for areas of poorer bone quality. Although macroporosity (pore size ~450 μm) has a strong impact on osteogenic outcomes, microporosity (pore size ~10 μm) and pore wall roughness play important roles as well. The HA ceramic rods with average pore size of 200 μm and smooth and dense pore walls failed to induce ectopic bone formation in dogs in contrast to rods made from the same material with average pore size of 400 μm but with rough and porous pore walls.

Microporosity results in larger surface area, which is believed to contribute to higher bone-inducing protein adsorption as well as to ion exchange and bone-like apatite formation by dissolution and reprecipitation.[66] The surface roughness enhances attachment, proliferation, and differentiation of anchorage-dependent bone-forming cells. The solid free form fabrication (SFF) technique allowed the fabrication of poly(desamino tyrosyl–tyrosine ethyl ester carbonate) (a tyrosine-derived pseudopolyamino acid) scaffolds with axial and radial channels and 500 μm pores separated by 500 μm solid walls or 80% porous walls.[66] Scaffolds from the same material with random pore distributions were used as controls in the *in vivo* experiments. Although there was no statistical difference in the bone formed in cranial defects in rabbits, bone ingrowth followed the architecture of the scaffolds. A continuous ingrowth from the outer periphery was observed in the scaffolds with random pore size, but scaffolds with same sized pores and solid walls promoted discontinuous ingrowth with bone islands throughout the entire scaffold. The scaffolds with same-sized pores and porous walls resulted in both types of bone ingrowths. It was hypothesized that discontinuous bone ingrowth may result in faster healing because bone forms not only from the margins but also throughout the entire

space of the defect.[67] These studies demonstrate the enhanced osteogenesis of porous versus solid implants, both at the macroscopic as well as the microscopic level.

4.6 OVERVIEW OF VARIOUS PROCESSING TECHNIQUES OF POROUS SCAFFOLD

A tissue-engineered scaffold must provide a germane environment for *in vitro* cell culturing in a bioreactor as well as providing a suitable environment after being implanted *in vivo*. These two environments differ in terms of nutrient concentration gradients, pressure gradients, and fluid velocities. *In vivo*, whereas diffusion is the primary mechanism for transporting nutrients, fluid flow is the principal mechanism for transport of nutrients and provision of mechanical stimuli *in vitro*.

For a scaffold to be considered successful, it is essential that it provides a nutrient-rich environment within the scaffold core for cells to lay down new matrix and minimize cell necrosis. The scaffolds with defined interconnected channels aid in the processes of cell nutrient delivery, waste removal, and vascular invasion.

Many of the conventional techniques yield scaffolds with random porous architectures, which do not necessarily produce a suitable homogeneous environment for bone formation (Fig. 4.7). Nonuniform microenvironments produce regions with

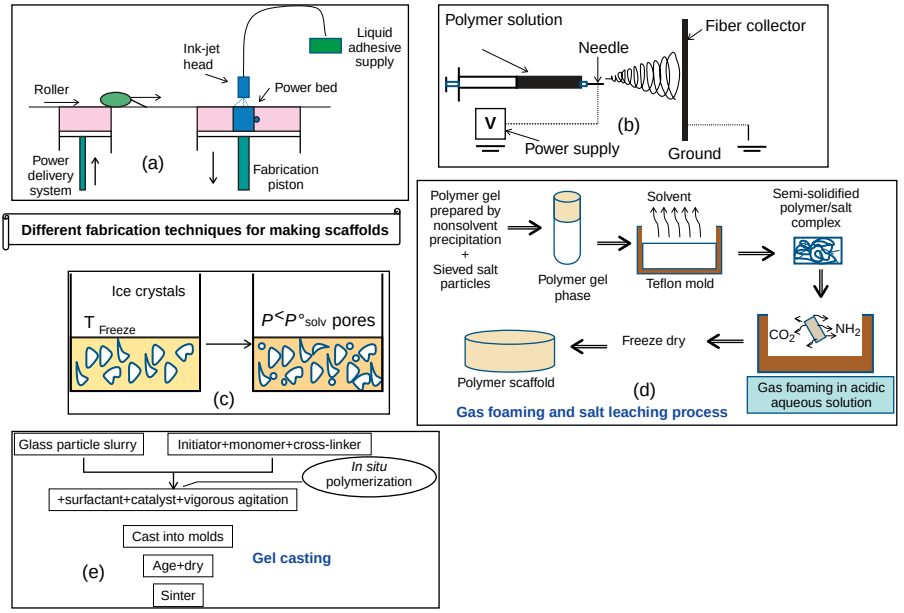

FIGURE 4.7 Schematic illustration of different fabrication process for making porous scaffolds for tissue engineering: rapid prototype three-dimensional printing (a), electrospining (b), Freeze casting (c), gas foaming and salt leaching (d), and gel-casting method (e).

insufficient nutrient concentrations, which can inhibit cellular activity and prevent the formation of new tissue. In the following discussion, some of the widely used processing routes are briefly described to illustrate how porous scaffolds can be fabricated.

Rapid prototyping is the most common name given to a host of related technologies that are used to fabricate physical objects directly from computer-aided design (CAD) data sources. These methods are unique in that they add and bond materials in layers to form objects. Such systems are also known by the names *additive manufacturing, additive fabrication, 3D printing* (Fig. 4.7a), *SFF, and layered manufacturing*. The advantages of this process are

1. Objects can be formed with any geometric complexity or intricacy without the need for elaborate machine setup or final assembly.
2. Rapid prototyping systems reduce the construction of complex objects to a manageable, straightforward, and relatively fast process.

A number of logical steps are to be sequentially followed in the 3D printing of solids. After providing the input based on CAD design, the loose powder is transferred from the powder delivery bed to the fabrication piston bed one layer at a time in the 3D printing setup. After each layer of loose powder has been transferred, an inkjet head (similar to an inkjet printer) dispenses a polymeric or liquid binder to only select areas, binding the powder in these areas and leaving the rest loose. After a layer is done, the fabrication piston moves down the platform, the roller spreads a new layer of loose powder over the previous layer, and the process repeats. The inkjet head is controlled by a computer that accepts CAD information, allowing superior control over the structure to be built. The loose powder from the previous layer acts as support material for the next layer, enabling overhanging structures to be built. After the build, the binder is cured at slightly elevated temperature, allowing all unbound powder to be removed by gentle agitation. Depending on powder and binders, a high-temperature heat treatment process sinters the bound particles, while the binder volatilizes, leaving a three-dimensional structure. Since the invention of 3DP process,[68–78] this process has been largely used to fabricate solid structures with different sizes and shapes with limited efforts in making porous materials.[79–81]

Electrospinning (Fig. 4.7b) is considered the most efficient technique for micro- and nanofiber production and one of the few processes to produce polymeric fibers on a large scale. Many applications of electrospinning are related to the biomedical field. In particular, electrospun polymeric fibers were used for the production of scaffolds for vascular tissue engineering[82–84] or hollow organ substitutes such as the bladder, trachea, and esophagus.[85–88] In this method, polymer solution is injected through a needle, which is maintained at a critical voltage (to create charge imbalance) and placed in the proximity to a grounded target. At critical voltage, charge imbalance begins to overcome the surface tension of the polymer fibers, forming an electrically charged jet. Grounded target is a rotating mandrel, which collects polymeric fibers.

Although requirements for medical scaffolds are numerous and vary with every application, some of them are fulfilled by the processing technique itself.[89] The architecture of the fibrous scaffold produced by electrospinning displays a high surface area for initial cell attachment and porosity for improved cell infiltration and nutrition diffusion, thus providing some key features of the native extracellular matrix.

Freeze casting (Fig. 4.7c) is a method in which rapid freezing of a colloidal stable suspension of HA particles in a nonporous mold takes place followed by sublimation of the frozen solvent under cold temperatures in vacuum. A different technique involving gas as a porogen has been introduced to develop porous scaffolds; this commonly known as *gas foaming* (Fig. 4.7d). The process begins with the formation of solid discs of poly(lactic-*co*-glycolic acid) (PGA), poly(L-lactic acid) (PLLA), or poly(lactic-*co*-glycolic acid) (PLGA) using compression molding with a heated mold. *Gel casting* (Fig. 4.7e), an advanced process for forming ceramics, was originally developed at ORNL to make complex-shaped automotive parts such as turbines. Gel casting is a wet ceramic-forming technique that involves the polymerization of a monomer in the presence of a solvent to form a rigid, ceramic-loaded body, which can be machined directly in a complex mold.[90] After gel formation, gel-cast green samples can be easily demolded and are then dried in controlled conditions.[91] The main advantage of this new process for making high-quality, complex-shaped ceramic parts is the lower cost compared with conventional forming techniques. In addition, gel casting appears attractive for an increasing number of applications ranging from accelerator magnets to artificial bone. A gel-cast part is soft enough to be machined quickly by less costly carbon steel tools.

Slip casting is a technique for making multiple, essentially identical parts inexpensively. Slip-casting methods provide superior surface quality, density, and uniformity in casting high-purity ceramic raw materials over other ceramic casting techniques, such as hydraulic casting, because the cast part is at a higher concentration of ceramic raw materials with little additives. A slip is a suspension of fine powders in a liquid such as water or alcohol with small amounts of secondary materials such as dispersants, surfactants, and binders. Early slip casting techniques used a plaster block or a flask mold. The plaster mold draws water from the poured slip to compact and form the casting at the mold surface. This forms a dense cast form, removing deleterious air gaps and minimizing shrinkage in the final sintering process.

A replication technique has also been used to prepare highly porous material with controllable pore sizes from inorganic materials and polymer materials. This technique is a multistep procedure in which first a replica of the porous structure is made from wax, polymer, or another material that can easily be removed by melting, burning, or dissolution. This replica is then used as a negative casting mold, and the interstices are filled with the desired polymer in the liquid phase. After hardening of the liquid polymer by curing, cooling, or precipitation, the mold forming the pore network is removed. A summary of conventional scaffold processing techniques as well as their advantages and disadvantages are summarized in Table 4.2.[92,93]

TABLE 4.2 Conventional Scaffold Processing Techniques for Tissue Engineering[93,94]

Process	Advantages	Disadvantages
Solvent casting and particulate leaching	Large range of pore sizes	Limited membrane thickness (3 mm)
	Independent control of porosity and pore size	Limited interconnectivity
	Crystallinity can be tailored	Residual porogens
	Highly porous structures	Poor control over internal architecture
Fiber bonding	High porosity	Limited range of polymers Residual solvents Lack of mechanical strength
Phase separation	Highly porous structures	Poor control over internal architecture
	Permits incorporation of bioactive agents	Limited range of pore sizes
Melt molding	Independent control of porosity and pore size	High temperature required for nonamorphous polymer
	Macro shape control	Residual porogens
Membrane lamination	Macro shape control	Lack of mechanical strength
	Independent control of porosity and pore size	Limited interconnectivity
Polymer–ceramic fiber composite foam	Independent control of porosity and pore size	Problems with residual solvent
	Superior compressive strength	Residual porogens
High-pressure processing	No organic solvents	Nonporous external surface Closed-pore structure
Freeze-drying	Highly porous structures High pore interconnectivity	Limited to small pore sizes
Hydrocarbon templating	No thickness limitation	Residual solvents
	Independent control of porosity and pore size	Residual porogens

4.7 OVERVIEW OF PHYSICOMECHANICAL PROPERTIES EVALUATION OF POROUS SCAFFOLD

Although increased porosity and pore size facilitate bone ingrowth, the result is a reduction in mechanical properties because this compromises the structural integrity of the scaffold. The increased porosity resulted in a higher median pore size (0.2–8.7 μm) and lower percentage of nanopores (<100 nm).[94] At the same time, lower compressive strength (37,000–430 kPa) and Weibull modulus (2.0–4.2) were reported.

Porous foams were fabricated by sintering poly(lactide-*co*-glycolide) microspheres. An increase in the microsphere diameter from 212–250 μm to 600 –710 μm resulted in larger median pore size (72–164 μm for 2 h of heating and 101–210 μm for 4 h of heating) and a wider pore distribution (38–110 μm in size, respectively) but had no effect

on total porosity. The compressive modulus was decreased from 297 to 232 MPa.[95] Similarly, higher porosity (80% vs. 58%) decreased mechanical properties of porous poly(L-lactide-*co*-D,L-lactide) scaffolds as compressive strength decreased from 11.0 to 2.7 MPa and modulus from 168.3 to 43.5 MPa.[96] The porosity of these scaffolds was ~80% because lower porosity resulted in less interconnected pores[97] and higher porosity results in low mechanical properties.[98] Higher porosity (48% vs. 44%) of cancellous structured titanium surface coating of dental implants resulted in lower tensile strength (16.1 vs. 31.7 MPa).[99] In general, the compromise in mechanical properties of the scaffold with increasing porosity sets an upper limit in terms of porosity and the pore size that can be tolerated.

Koc et al.[100] fabricated and characterized porous tricalcium ceramics using a modified slip-casting technique. The slip was prepared by suspending custom-made tricalcium phosphate (TCP) powder and poly(methyl methacrylate) (PMMA) beads in an aqueous medium stabilized with an acrylic deflocculant. Porous TCP ceramics were obtained by sintering the polymer-free preforms for 2 h at 1000°C. The ceramic was prepared from a casting slip, which contained 70% polymer beads in the size range of 210–250 μm. The average size of large pores in the sintered ceramic was around 190 μm. Koc et al.[100] suggested that higher proportions of polymer beads in slip solids led to the development of highly porous ceramics with thinner walls. As the amount of polymer beads was raised, the size of interconnections increased proportionately. It was concluded that porosity network of this nature would allow free circulation of body fluids.

Li et al.[101] studied novel method to manufacture porous hydroxyapatite by dual phase mixing. Their technique was based on mixing the immiscible phases of HA slurry and PMMA resin. Naphthalene particles were embedded to get >50% porosity. The majority of pores could be located within the range of 200–300 μm for HA with 50% porosity. The average compressive strength was reported as 8.9 MPa for 50% porous HA and was only 4.8 MPa for HA with 60% porosity. They also concluded that by controlling the process parameters such as the viscosity of HA slurry, the HA–PMMA ratio or the mixing time and speed, it is possible to adjust porosity, pore size, and interconnectivity.

Uniform porous hydroxyapatite scaffolds were also prepared by using another solid phase, which was completely burnt out at the time of sintering. Polystyrene microspheres were used by Tang et al.,[102] and they developed HA material of varying diameter and porosity (diameter, 436 ± 25 nm, 892 ± 20 nm, and 1890 ± 20 nm; porosity, 46.5%, 41.3%, and 34.7%, respectively). On the other hand, Itatani et al.[103] used H_2O_2 as a foaming agent and found that by changing the concentration of H_2O_2 solution from 0 to 20 mass%, HA compact exhibited pore sizes with maximum porosity (71.7%) at around 0.7 μm, 5–100 μm, and 100–200 μm. In a different work, Thijs et al.[104] studied a novel technique to produce macroporous ceramics using seeds and peas as sacrificial core materials. The first step in this technique was to coat the seeds and peas with wetting ceramic slurry that undergoes gelation. The coated seeds and peas were consolidated by packing them in a container and infiltrating with ceramic slurry, which underwent gelation. The compacts thus obtained were subjected to the conventional steps of drying, binder burnout, and sintering. The

resulting bodies had greater than 90% porosity with pore size determined by the size of the seeds or peas.

The polymer replication or sponge technique is another commonly used process to develop porous scaffolds for artificial bone applications. Ramay et al.[105] developed the porous HA scaffold having apparent densities of 0.04–0.78 g/cm^3 and compressive strength of 0.55–5 MPa (Figs. 4.8a and 4.9). Similarly, Sopyan et al.[106] adopted a similar processing route and reported enhanced properties. They reported the compressive strength ranging from 1.3 to 10.5 MPa for the increased apparent density from 1.27 to 2.01 g/cm^3. It was concluded that the homogeneity of slurry and the effect of heating rate on porosity and density of porous bodies in turn influenced the compressive strength. More homogeneous slurries and a faster heating rate gave porous bodies with the increased compressive strength caused by a higher apparent density and crystallinity.

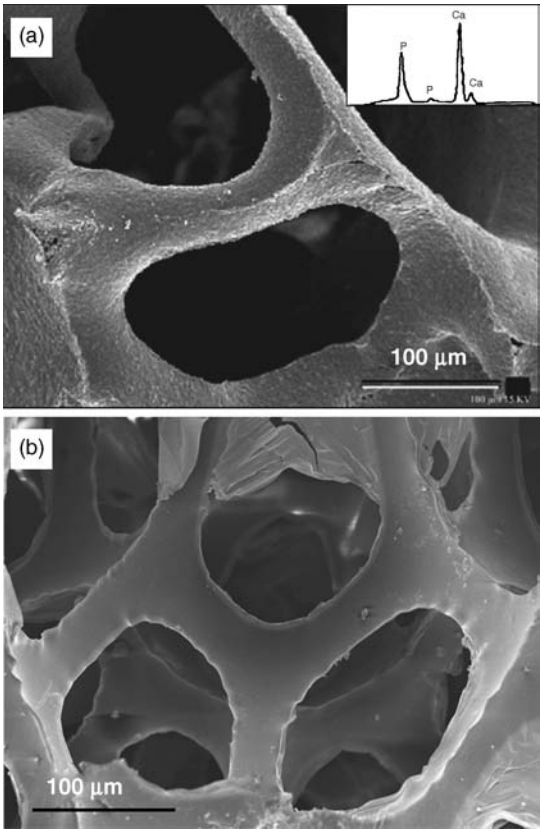

FIGURE 4.8 Scanning electron microscope (SEM) micrographs of an HA scaffold showing the sintered structure of the pore walls and pores. (a) At struts with EDS spectrum as an inset showing the amount of Ca and P present in the sintered scaffold. Printed with permission from Ref. 129. (b) SEM micrograph of an HA scaffold sol-gel derived HA powder and polymer slurry. Printed with permission from Ref. 129.

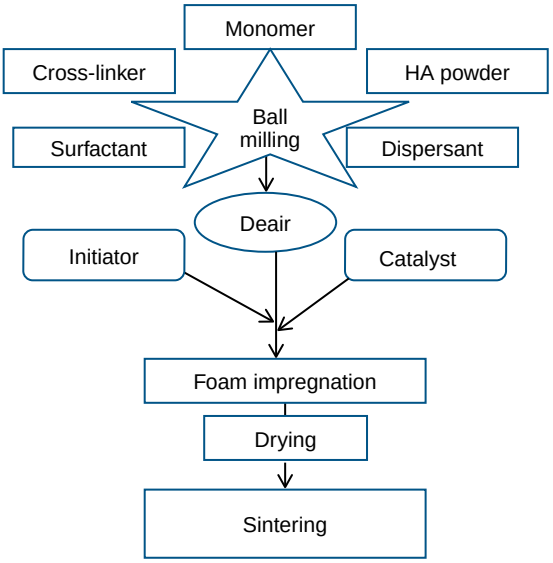

FIGURE 4.9 A flow chart of process steps for scaffold fabrication using combined gel-casting and polymer sponge methods. Idea adapted from Ref. 105.

The present authors[107] also developed the macroporous HA scaffolds using polymer sponge replication method with interconnected oval-shaped pores of 100–300 μm with a pore wall thickness of ~50 μm (Fig. 4.8b). The obtained compressive strength of 60 wt% HA loaded scaffold was calculated 1.3 MPa. The biological response of the scaffold was investigated using human osteoblast-like $SaOS_2$ cells. Their results showed that $SaOS_2$ cells were able to adhere, proliferate, and migrate into pores of scaffold. Furthermore, the cell viability was found to increase on porous scaffold compared with dense HA. They also investigated the expression of alkaline phosphate and concluded that the differentiation marker for $SaOS_2$ cells was enhanced for porous HA scaffold compared with nonporous HA disc with respect to the number of days of culture (Fig. 4.10).

Successful fabrication of porous bioceramic using polyurethane (PU) sponge was reported by Soon-Ho Kwon et al.[108] Porosity was controlled by the number of coatings on the sponge struts. Single coating results in a porosity of ~90%, where as five-layered coating gave rise to 65% porosity. In both cases, the pores were completely interconnected. The compressive strength was strongly dependent on the porosity and weakly dependent on the type of ceramics: HA, TCP, or HA–TCP composite. At 65% porosity level, the strength was ~3 MPa. The TCP exhibited the highest dissolution rate in a Ringer's solution, while HA had the lowest rate. The biphasic HA–TCP composite showed an intermediate dissolution rate. The biodegradation of calcium phosphate ceramics could be controlled by simply adjusting the amount of HA or TCP in the ceramic.

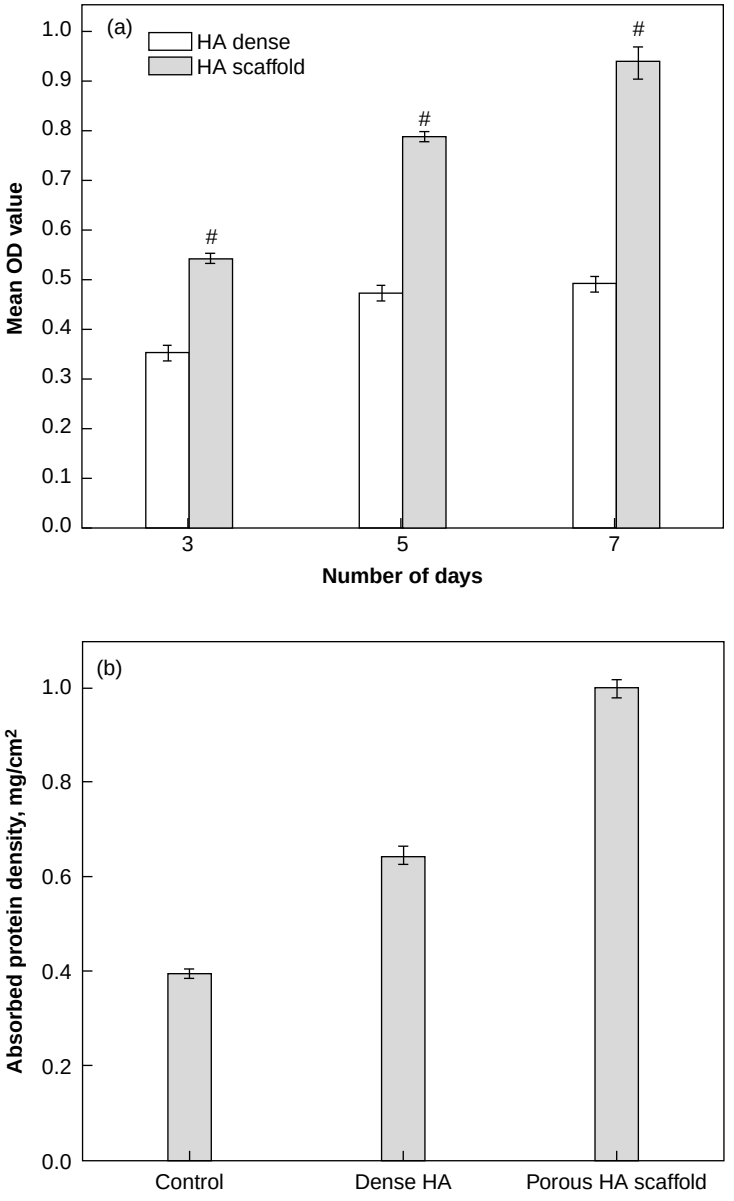

FIGURE 4.10 (a) ALP assay results showing the $SaOS_2$ cell response on dense HA and microporous HA scaffold after 3, 5, and 7 days of culture. *Asterisks* represent significant difference at $P < 0.05$ with respect to compositions, and error bars correspond to $+/-$ 1.00 SE for number of days of culture. (b) Comparison of BSA protein absorption behavior of scaffold with dense HA and a negative control disc after incubation for 4 h. Idea adapted from Ref. 107.

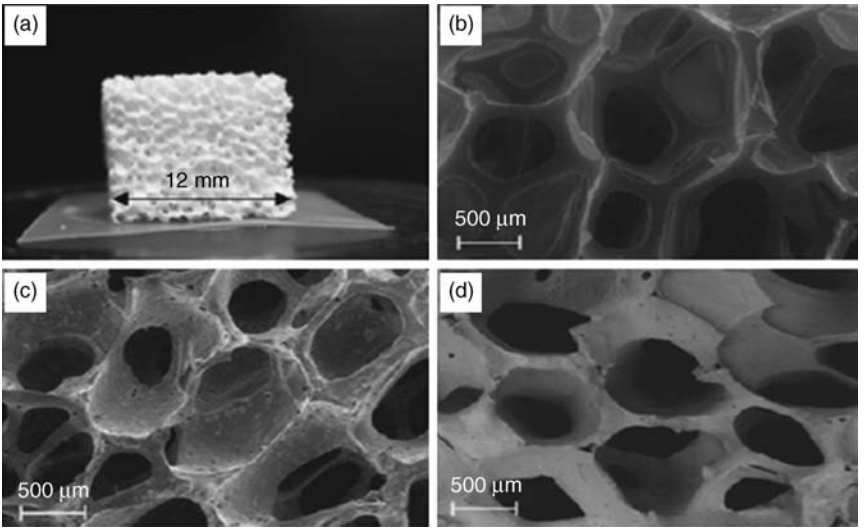

FIGURE 4.11 A macrograph of a porous scaffold (a), SEM image of the macrostructure of the sponge (b) and of the scaffolds sintered at 1300°C with the in-laboratory synthesized powder (c), and the commercial powder (d). The porosity is open and highly interconnected in both of the samples. Idea adapted from Ref. 109.

Gervaso et al.[109] produced porous scaffolds with a polymer sponge templating method using reactive submicrometer powders synthesized by a hydroxide precipitation sol–gel route. The templating method ensured a highly interconnected macrochanneled porous structure with a more than 500 μm mean pore size and 90% porosity (Fig. 4.11). The high reactivity of the powder led to an efficient sintering mechanism with a high and crack-free linear shrinkage ($19 \pm 2\%$) and a significant BET-specific surface area reduction (from 12 to 0.33 m^2/g). The powder does not dissociate into secondary phases during sintering. Despite the extreme porosity, the scaffolds had high mechanical performance (compressive strength ~0.51 MPa; Weibull modulus~4.15) compared with similarly prepared scaffolds from high-quality commercial HA powder (Fig. 4.12).

The slurry infiltration process for making porous ceramics was studied by Schwartzwalder and Somers.[110] In this process, PU foam was infiltrated with ceramic slurry, and the body was compressed by passing it through a set of rollers to remove the excess slurry. In this manner, the slurry remained coated on the PU struts, and open pore channels were left in between. The coated PU foam was then dried followed by burnout of the PU and sintering at a higher temperature. The foams produced were reticulated foams with porosity within the range of 75–90%.

Zhu et al.[111] investigated the influence of the compressive strain during roll pressing and the number of passes on the foam microstructure. It was seen that the quality of slurry coating on to PU struts was strongly dependent on the magnitude of compressive strain rather than the number of passes. Higher compressive strain resulted in thinner slurry coating on the struts and a lower bulk density. The coating

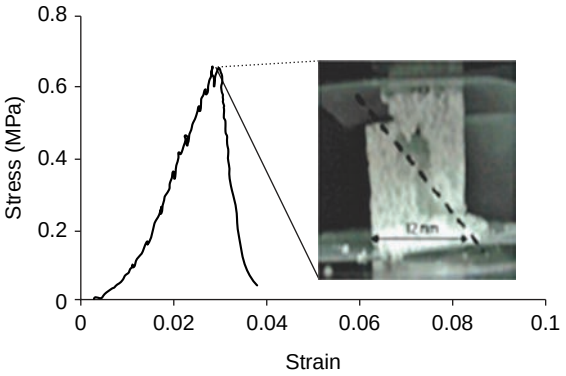

FIGURE 4.12 Stress–strain curve of an SL scaffold subject to compression test. In the inset is a picture of the failure of the sample that occurs at the peak of the stress. Idea adapted from Ref. 109.

of slurry onto PU struts was also affected by slurry viscosity. Highly fluid slurries were not very effective in coating the PU foam struts, resulting in accumulation of slurry at the bottom of PU foam. On the other hand, Pu et al.[112] pointed out that the conventional roll-pressing procedure results in accumulation of slurries at the joint of the polymeric struts. Lin et al.[113] focused on the preparation of macroporous calcium silicate ceramics using PEG as a pore former. The sintered compacts with porosity in the range of 40–75% have been obtained by varying the amount and size of ceramic and PEG particles and the sintering temperature. The molecular weight of PEG plays an important role in the morphology, structure, and the pore size of the microporous calcium silicate. Also, PEG plays a main role in larger pore formation when enough mass of PEG with lower molecular weight is added.

A novel combination of PU foam method and a hydrogen peroxide (H_2O_2) foaming method was used to fabricate the macroporous HA scaffolds.[114] Such scaffolds have a unique macroporous structure and special struts of polymer–ceramic interpenetrating composites. Micropores were present in the resulting porous HA ceramics after infiltration with PLGA polymer. The internal surfaces of the macropores were further coated with a PLGA bioactive glass composite. It was found that the HA scaffolds fabricated by the combined method show high porosities of 61–65% and proper macropore sizes of 200–600 μm. The PLGA infiltration improved the compressive strengths of the scaffolds from 1.5–1.8 MPa to 4.0–5.8 MPa.

Similarly, Narbat et al.[115] fabricated porous HA–gelatin composite scaffolds. They reported that the prepared scaffold has an open, interconnected porous structure with the pore size of 80–400 μm, which is suitable for osteoblast cell proliferation. The mechanical properties of the scaffolds with different weight fraction of HA (30, 40, and 50 wt%) was assessed, and it was found that the gelatin–HA with a ratio of 50 wt% HA has the compressive modulus of ∼10 GPa, the ultimate compressive strength of ∼32 MPa. The porosity and the apparent density of 50 wt% HA scaffold were calculated, and it was found that the addition of HA could reduce the water absorption and the porosity.

The demonstration of potato starch as both a consolidator or binder and a pore former in forming porous ceramics was reported by Lyckfeldt and Ferreira.[116] In this process, 16–60% starch was added as dry powder weight basis to ceramic slips and homogenized for 2 h. The homogenized slip was consolidated in a mold at 80°C followed by drying, binder burnout, and finally sintering. The pore sizes in the range 10–80 μm and porosity between 23% and 70% were obtained by varying ceramic loading and the nature and amount of starch. Importantly, increasing the amount of a specific type of starch resulted in a large pore size because of a greater degree of contact among the starch particles.

The freeze-casting route can produce porous HA scaffolds with porosities in the range of 40–60%.[117] By adopting this route, the pores were open and unidirectional and exhibited a lamellar morphology. Such a porous scaffold has a compressive strength of 145 MPa. Potoczek[118] studied the gel casting of HA foams using agarose as gelling agent. The viscosity of the slurries could be adjusted by agarose concentration and HA solid loading. These parameters were essential in tailoring the porosity as well as the cell and window sizes of the resulted HA foams. Depending on HA solid loading (24–29 vol%) and agarose concentration (1.1–1.5 wt% with regard to water) in the starting slurry, the mean cell size ranged from 130 to 380 μm, and the mean window size varied from 37 to 104 μm. Depending on the porosity range (73–92%) and the mean cell and window size, the compressive strength of HA foams was found to be in the range of 0.8–5.9 MPa.

Chloroform as a binder was used to fabricate porous scaffold in a 3DP route by Giordano et al.[119] They studied the mechanical properties of 3DP processed PLLAparts. Test bars were fabricated from low- and high-molecular-weight PLLA powders. The binder printed per unit length of the powder was varied to analyze the effects of printing conditions on mechanical and physical properties of the PLLA bars. The maximum measured tensile strength for the low-molecular-weight PLLA (53,000 g/molecule) was 17.4 ± 0.7 MPa and for high-molecular-weight PLLA (312,000 g/molecule) was 15.9 ± 1.5 MPa. Kim et al.[120] evaluated the survival and function of hepatocytes on a scaffold with an intrinsic network of interconnected channels under continuous flow conditions. The scaffolds were designed and fabricated using the technique of 3DP on copolymers of polylactide–coglycolide (PLGA 85:15). 3DP was also used to selectively direct a solvent onto PLGA powder particles packed with sodium chloride particles (45–150 μm). The polymer scaffolds were fabricated in the shape of a cylinder of 8 mm in diameter and 7 mm in height. They contained 12 interconnected longitudinal channels (800 μm in diameter) running through the length of the scaffold and 24 interconnected radial channels (800 μm diameter) at various lengths of the devices. The salt crystals were leached out to yield porous devices of 60% porosity with micropores of 45–150 μm in diameter. The fabrication of scaffolds for tissue engineering requires choosing a conformation method that yields pieces with interconnected porosity and pores in the 20–400 μm range.[121]

Table 4.3 lists some selected typical physical and mechanical properties of scaffolds obtained by various processing routes. A similar material was processed under different processing techniques, which result in varying physical properties.

TABLE 4.3 An Overview of Various Processing Techniques Used to Develop Porous Scaffolds with Varying Pore Sizes and Properties

Technique	Material	Open Porosity	Pore Size or Dimension (μm)	Compressive Strength (MPa)	Reference
Freeze casting	HA	47–52	5–30	12–18	[124]
	HA	50–60	80–110	7.5–20	[125]
	HA	40–65	20	40–145	[122,123]
	HA–polycaprolactone	87	150–200	–	[126]
	HA, PHBHH*x*, dioxane	88–92	–	–	[127]
Polymer sponge	HA	86	420–560	0.21	[128]
	Glass-reinforced HA	85–97.5	420–560	0.01–0.18	[129]
	HA	70–77	200–400	0.55–5.0	[130]
	45S2 bioglass	89–92	510–720	0.27–0.42	[131]
	HA	–	100–200	–	[132]
	HA	–	100–400	–	[133]
	HA-TCP	36	100–150	–	[134]
	$Ca_2MgSi_2O_7$	63–90	300–500	0.53–1.13	[135]
	HA, polyvinyl alcohol, acetone	85	–	–	[136]
Gel casting	HA	76–80	20–1000	4.4–7.4	[137]
	HA	72–90	17–122	1.6–5.8	[138]
	HA–PMMA		200–400	5.0	[139]
Slip casting	13–93 glass	40–45	100–300	21–23	[140]
	HA	85	200–500	1.09–1.76	[141]
Gas foaming	PLGA	85–96	193–439	0.16–0.29	[142]
	n-HA/PU	80	100–800	0.27	[143]
Salt leaching	TCP cement calcium metaphosphate	–	200	–	[144]

HA, hydroxyapatite; PLGA, poly(lactic-*co*-glycolic acid; PMMA, polymethylmethacrylate; PU, polyurethane; TCP, tricalcium phosphate.

It can be summarized from Table 4.3 that the variation in pore size and open porosity directly affects the mechanical behavior of the prepared scaffold. The freeze-casting method provides the highest compressive strength of around 40–145 MPa. The lower strength (<1 MPa) of porous constructs was obtained in the polymer sponge method and gas-foaming process. Recent investigations have shown that porous HA scaffolds, with a lamellar-type microstructure and unidirectional pores, can be obtained by freeze casting of aqueous suspensions.[122,123]

4.8 OVERVIEW OF BIOCOMPATIBILITY PROPERTIES: EVALUATION OF POROUS SCAFFOLDS

Karageorgiou and Kaplan[145] investigated the influence of porosity on osteogenesis in three-dimensional biomaterial scaffolds. It has been seen that the porosity and pore size of biomaterial scaffolds play critical roles in bone formation *in vitro* and *in vivo*. The minimum requirement for pore size is considered to be ~100 μm because of cell size and migration requirements. However, pore sizes of ~300 μm are recommended because of enhanced new bone formation and the formation of capillaries. The effect of these morphological features on osteogenesis *in vitro* and *in vivo*, as well as relationships to mechanical properties of the scaffolds, was addressed. *In vitro*, lower porosity stimulates osteogenesis by suppressing cell proliferation and forcing cell aggregation. In disparity, higher porosity and pore size result in greater bone ingrowth *in vivo*.

The kinetics of bone-like apatite formation on sintered hydroxyapatite in a simulated body fluid was studied by Kim et al.[146] The surfaces of two HAs, which have been sintered at different temperatures of 800°C and 1200°C, were investigated as a function of soaking time in simulated body fluid (SBF) using transmission electron microscopy (TEM) attached with energy-dispersive spectrometry (EDX) and laser electrophoresis spectroscopy.

The synthesis of biomimetic Ca-hydroxyapatite powders at 37°C in synthetic body fluids was reported by Tas.[147] Initially, HA was prepared as a nanosized (~50 nm), homogeneous, and high-purity ceramic powder from calcium nitrate tetrahydrate and diammonium hydrogen phosphate salts dissolved in modified SBF solutions at 37°C and a pH of 7.4 using a novel chemical precipitation technique. The synthesized precursors were found to reach a phase purity of 99% easily after 6 h of calcination in air atmosphere at 900°C after oven-drying at 80°C.

Biocompatibility and osteogenicity of degradable Ca-deficient hydroxyapatite (CDHA) scaffolds were investigated by Guo and coworkers.[148] They made scaffold from calcium phosphate cement for bone tissue engineering with a particle-leaching method. They demonstrated that the CDHA scaffolds with porosity of 81% showed open macropores with pore sizes of 400–500 μm. Thirty-six percent of these CDHA scaffolds were degraded after 12 weeks in Tris–HCl solution. The results revealed that the CDHA scaffolds were biocompatible and had no negative effects on the mesenchymal stem cells (MSCs) *in vitro*. The CDHA scaffold, after 8 week implantation in rabbit model shows good *in vivo* biocompatibility and extensive osteoconductivity.

Park et al.[149] reported the fabrication of patterned PLLA substrates with the spatial organization of cells obtained using the 3DP route. They demonstrated an integration of polymer processing and selective polymer surface modification using methods suitable for construction of three-dimensional polymer scaffolds, which may aid such cell organization. They concluded that their approach may be generally useful for creating regionally selective, microarchitectured scaffolds fabricated from biodegradable polymers for spatial organization of diverse cell types.

Peng et al.[150] developed a novel scaffold with large dimension of 3–4 cm in length and 1–1.5 cm in diameter. They designed and fabricated the scaffold for bone tissue engineering *in vivo*. Porous HA in the form of a tube coated with a thin layer of poly(L-lactic acid) (PLA) held the HA spherules together and provided the initial strength of scaffolds. Studies on engineering of large bone tissue were underway by use of the hybrid scaffold implanted at different nonrepairing sites such as muscle, peritoneum, and bone *in vivo*. The novel scaffolds were implanted in different sites of dogs (Fig. 4.13). To compare the influence of the distribution of biological substance on the osteogenesis, the HA spherules were mixed homogenously with comminuted bone granules before filling in the porous HA tube. The primary tissue section showed a promising new bone growth induced by the homogeneous addition of comminuted bone granules.

Becker et al.[151] evaluated the ability of CAD synthetic hydroxyapatite and tricalcium phosphate blocks to serve as scaffolds for intramuscular bone induction

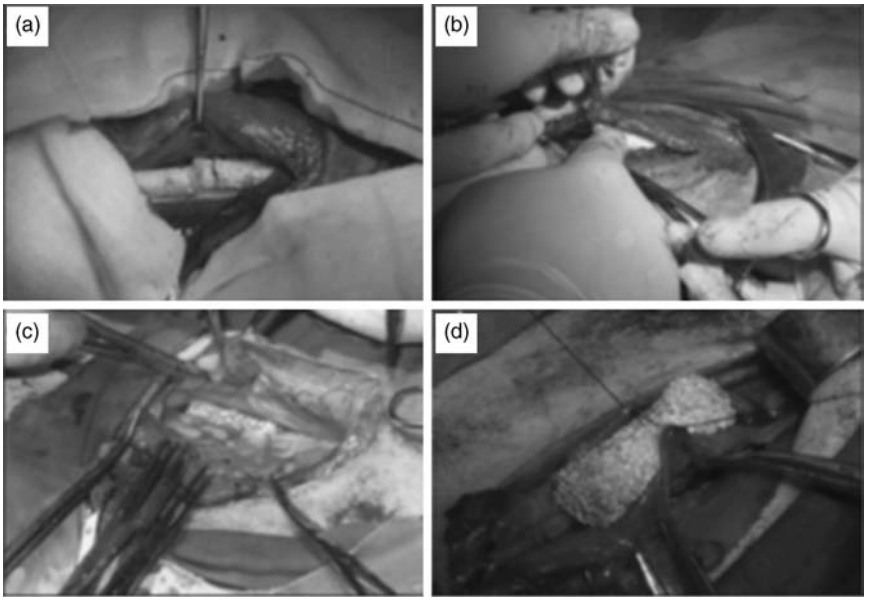

FIGURE 4.13 Digital photos showing the implantation of the porous scaffold at different sites of natural bone: beside the femur (a), in the muscle (b), in the abdominal cavity (c), and in the peritoneum pocket (d). Printed with permission from Ref. 129.

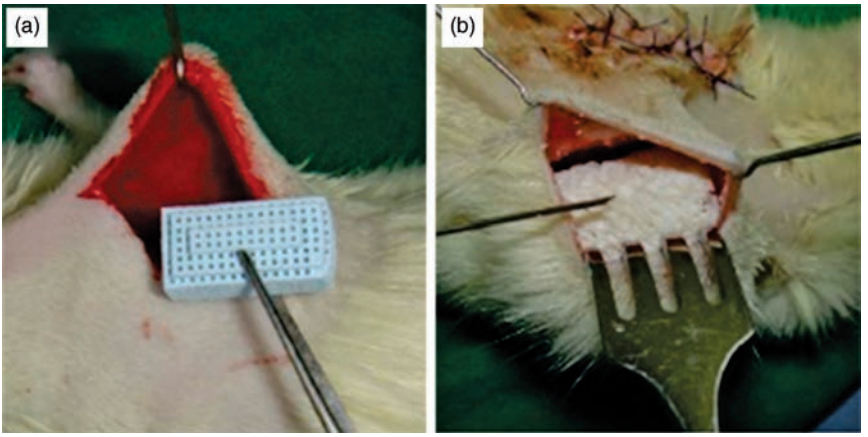

FIGURE 4.14 Digital photographs illustrating (a) insertion of a hydroxyapatite block into the pouch. (b) A bovine HA block was placed into the pouch on the right side. Printed with permission from Ref. 130.

in a rat model (Fig. 4.14). Individually, they kept the designed 3D-printed rounded and porous HA and TCP blocks in pouches in the musculus latissimus dorsi in 12 Lewis rats bilaterally. For 8 weeks, the bone generation was monitored by computed tomography and fluorescence labeling. For all scaffolds, toluidine staining revealed vital bone directly on the scaffold materials but also in the gaps between the walls of interconnected pores. They concluded that the specially shaped HA and TCP blocks tested against the bovine HA blocks could exhibit good biocompatibility and osteoinductivity *in vivo*.

The present authors also developed porous HA scaffolds using the polymer blend method.[152] In this method, PMMA was used as porogenous template to obtain micro- and mesoporosity. The pore size in the sintered ceramics was in the range of 1–50 μm. The cell adhesion test with human osteoblast cells ($SaOS_2$) confirmed good cytocompatibility of porous composite. Fluorescent staining of osteoblast revealed a well-developed cytoskeleton with strong stress fibers (Fig. 4.15). The ALP activity of osteoblast-like cells grown on the porous scaffolds for various culture times was significantly higher than that of dense HA. The results suggested that the porous HA–PMMA hybrid composite can be used as substrate, which should facilitate better cell differentiation than sintered HA. It is consistent with the previous reports that the polymer–HA scaffolds are superior to the pure polymer scaffolds for osseous tissue engineering[153] because the presence of HA hydroxyl groups could promote calcium and phosphate precipitation and improve interactions with osteoblasts.[154] It was concluded that such a processing approach offers a better possibility to produce porous HA scaffolds with micro- and mesopores, which can stimulate significant cell adhesion and osteoblast differentiation.

Kwon et al.[155] successfully fabricated porous bioceramics with varying porosity, using the PU sponge technique. When a porous solid was produced by a single coating, the porosity was ∼90%, and the pores were completely interconnected.

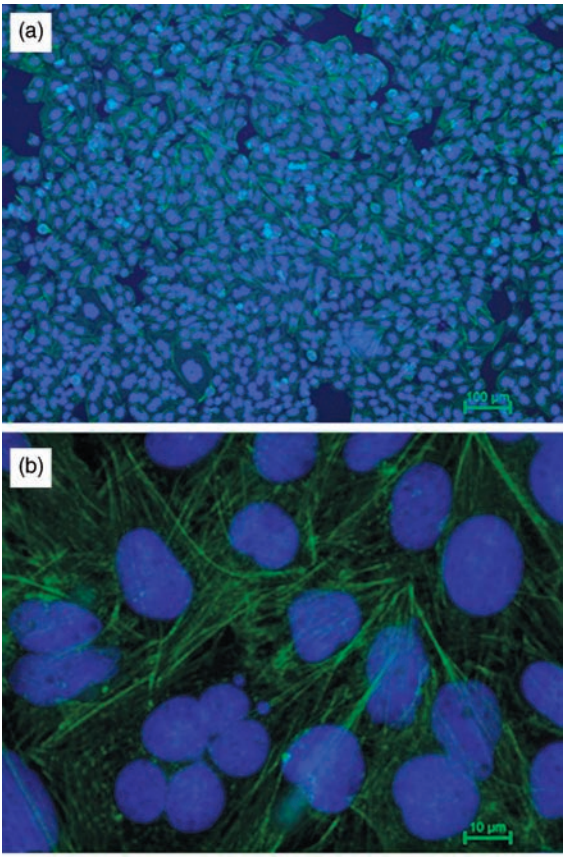

FIGURE 4.15 Fluorescent imaging of osteoblast cells revealing a well-developed cytoskeleton with strong actin stress fibers oriented in the adhered cells in their longitudinal direction.

When the sintered ceramic was coated five times after the porous network had been made, the porosity decreased to 65%. The compressive strength was strongly dependent on the porosity and weakly dependent on the type of ceramics (i.e., HA, TCP, or HA–TCP composite). At a 65% porosity level, the strength was $\sim$3 MPa. The TCP exhibited the highest dissolution rate in Ringer's solution with HA exhibiting the lowest rate. The biphasic HA–TCP composite showed an intermediate dissolution rate. The biodegradation of calcium phosphate ceramics could be controlled by simply adjusting the amount of HA or TCP in the ceramic.

4.9 OUTSTANDING ISSUES

Although available literature, as summarized above, provides details on synthesis, properties and different applications of porous scaffolds, it is quite clear that porous

material with desired porous architecture for specific biomedical application is still awaiting. Regardless of the type of porous scaffold (ceramic or polymer based), all tissue engineering scaffolds should meet the following requirements:

1. Surface wettability properties to promote cell adhesion, proliferation, and differentiation.
2. Mechanical properties to withstand stress.
3. Large ratio of surface area to volume to allow tissue ingrowth.
4. Controlled rate of degradation (particularly for polymer scaffolds).

In this chapter, emphasis has also been placed on the design of polymeric scaffold materials that obtain specific, desired, and timely responses from surrounding cells and tissues. The overall challenges concerning critical scaffold design parameters include polymer assembly, surface properties, nano- or macrostructure, biocompatibility, biodegradability, and mechanical properties.

From the discussion on porous scaffolds, it should be clear that multiscale porous scaffolds would be an interesting material to be developed and investigated in the future. It is known that microporosity with pore sizes of less than 1 μm helps in initial protein adsorption, and pore sizes of 1–20 μm aid in cell attachment as well as oriented cellular growth at the initial stage of cell proliferation and growth. Also, macroporosity with pore sizes of 100–1000 μm facilitates tissue or bone ingrowth *in vivo*. It would be therefore ideal to produce a porous scaffold with top surface of less than 1 μm pore size and of bioresorbable material followed by pores of 1–20 μm size and subsequently pores of 100 μm or larger with a top-down approach. Although the fabrication of scaffolds with such a controlled or gradient pore size could be a major challenge in terms of processing, one can use 3D printing method to produce such gradient porosity in HA–TCP or TCP–Ti system. As mentioned earlier, the type and amount of binder as well as postprinting heat treatment would be related challenges.

The potential for improving the mechanical properties of bioceramics or polymer composite scaffolds with a fabrication approach has been demonstrated in several systems with limited success to achieve mechanical properties, particularly compression strength, or modulus in the range of values for cancellous bone. All of the processing approaches can be conveniently classified into two categories: (1) chemical precursor-based routes and (2) engineering-based approaches. Although the first category largely results in uncontrolled porosity with heterogeneous or untailored pore sizes, the second category (i.e., 3D printing or other rapid prototyping routes) produces porous scaffolds with tailored porosity. More emphasize should be placed in the future to develop porous scaffolds with properties comparable to those of cancellous bone.

Another key question that needs to be addressed in future research is whether porosity in inherently bioinert scaffold material can induce bioactivity. To illustrate this issue, one can do *in vitro* or *in vivo* experiments on porous Ti and porous HA under identical conditions with similar porous architecture.

4.10 CONCLUSIONS

This chapter has provided an overview of the processing and physical and biological properties that play important roles in the design of porous ceramics. Various illustrative examples of porous scaffolds have also been discussed in detail.

In summary, the design criteria and critical issues in developing porous scaffolds for bone tissue engineering have been discussed. In addition, the suitability of porous scaffolds for both *in vitro* and *in vivo* biocompatibility properties has been rationalized. Various techniques for developing scaffolds along with their advantages and disadvantages are discussed. A high degree of interconnectivity in conjunction with a suitable pore size has been emphasized for porous scaffolds to minimize diffusion and pore occlusion. However, there is a limited understanding in terms of the long-term *in vitro* and *in vivo* biocompatibility properties of porous scaffolds. In particular, the degradation and ion-release kinetics of inorganic phases from highly porous systems. For bone regeneration, the utmost challenge for porous scaffolds is to impart the mechanical strength for replacing the bone defects as well as efficient load transmission. Despite the availability of a number of fabrication techniques, the aspects of mechanical reliability of scaffolds together with induction of vascularization and tailored degradability are yet to be addressed. At present, none of the available fabrication routes offers such a combination of properties in a designed porous scaffold. Reviewing the experimental and clinical studies, it can be concluded that an ideal scaffold for tissue-engineered bone and cartilage has not yet been developed. In general, the scaffolds require individual external shape and well-defined internal structure with interconnected porosity to host most cell types. From a biological point of view, the designed matrix should serve various functions, including (1) as an immobilization site for transplanted cells; (2) formation of a protective space to prevent unwanted tissue growth into the wound bed and allow healing with differentiated tissue; and (3) directing migration or growth of cells via surface properties of the scaffold or via release of soluble molecules such as growth factors, hormones, or cytokines. At the closure, it needs to be emphasized that future studies should concentrate more on adopting engineering-based processing approaches to fabricate porous scaffolds with tailored porosity and to develop a comprehensive understanding of relationships among processing, microstructure, biocompatibility, and clinical performance. Toward this, a battery of *in vitro* biochemical assays to evaluate porosity dependence of cell fate process as well as long-term *in vivo* biocompatibility assessment in suitable animal model together with investigation on bone regeneration using microcomputer tomography as well as TEM of bone-implant interface are to be performed. In addition, molecular biology techniques, such as flow cytometry, need to be used to quantifically assess the cell proliferation, cell cycle and cell apoptosis or reactive oxygen stress (ROS) generation for specific cell types when grown on porous scaffolds.

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5

SYNTHETIC ENROUTES TO ENGINEER ELECTROSPUN SCAFFOLDS FOR STEM CELLS AND TISSUE REGENERATION

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5.1 INTRODUCTION

Nanotechnology is one of the rapidly growing scientific disciplines involved in the development of materials with nanoscale dimensions, and it aims at resolving many of the diseases related to organ damage. Nanotechnology for tissue engineering application focuses on the role of extracellular matrix (ECM) in cell patterning, migration, proliferation, and differentiation.[1] Tissue growth or regeneration is achieved by stimulation assisted by cells or drug or growth factor loaded matrix at the damage tissue site. Matrix suitable for tissue regeneration should satisfy a few criteria such that it should be physically stable within the implanted site of injury, direct and control tissue growth, be biodegradable *in vivo*, and should not produce toxic metabolic byproducts. Various biomimetic tissue engineering scaffolds are made from natural and synthetic polymers possess certain optimal mechanical strength and form a sponge type or nanofibrous matrix or hydrogel architecture.

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To engineer such complex and multifunctional scaffolds, many developments in the field of nanotechnology were evolved to create porous, nanometer-sized nanofiber scaffolds so as to determine the fate of the cells, allow regulation of specific protein expression patterns, and encourage cell-specific scaffold remodeling. These nanotechniques can modulate surface topography down to submicron or nanometer range, and they include methods such as nanoscale surface pattern fabrication, electrospinning, and self-assembly fabrication.[2] Incorporating biological signals in the form of growth factors, angiogenic factors, cell surface receptors, drug entities, reactive oxygen species, and spatial cues can further influence cell proliferation, migration, differentiation, and 3D organization.

Nanofibrous scaffolds are ideal for the purpose of tissue regeneration because their dimensions are similar to components of ECM and mimic its fibrillar structure, providing essential cues for cellular organization and survival function. Electrospinning is one of the most important promising techniques for designing polymer nanofibers for tissue engineering applications. Tissue engineering is a multidisciplinary area of research and clinical application that aims for the repair, replacement, or regeneration of cells, tissues, or organs to restore impaired function owing to congenital defects, disease, trauma, or aging. The principle of tissue engineering and regenerative medicine is the application opt biomaterial scaffolds to produce living structures with sufficient size and function to improve human lives. The native ECM is a complex arrangement of proteins and polysaccharides such as collagen, hyaluronic acid, proteoglycans, glycosaminoglycans, and elastin, and electrospinning produces nanofibers with ECM mimicking molecules and architecture This chapter discusses "electrospinning process" (Fig. 5.1) as a novel method for engineering scaffolds for stem cells and tissue regeneration. Scaffolds made of natural proteins and carbohydrate materials have poor mechanical properties, and in

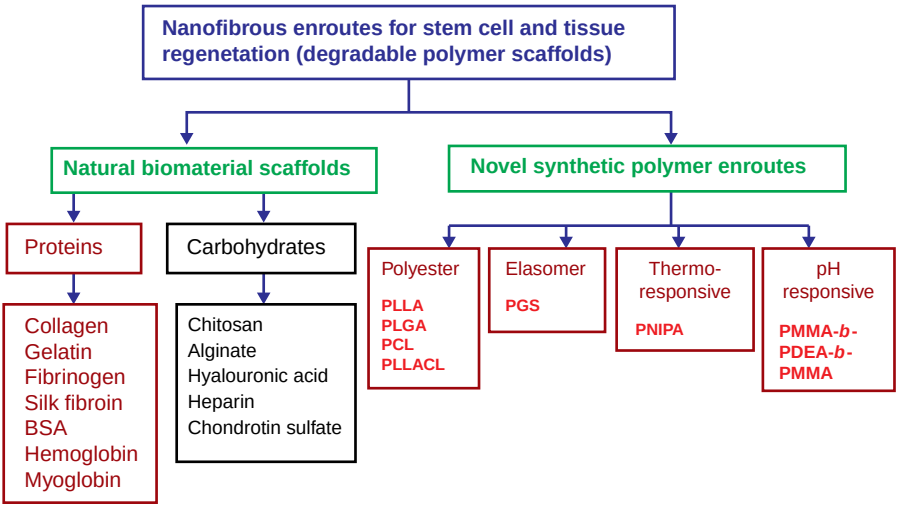

FIGURE 5.1 Biodegradable nanofiber scaffolds for tissue engineering applications.

most cases, they cannot be applied for tissue engineering. Cross-linking is carried out by many researchers to maintain the structural integrity of the construct.

To improve the stability of the natural protein[3–24] or carbohydrate-based scaffolds and to reduce the biodegradation rate of the scaffolds, cross-linking becomes inevitable. The details of electrospun cross-linked polymeric scaffolds used for tissue regeneration are also provided in this chapter.

5.1.1 Electrospun Nanofibrous Scaffolds for Tissue Engineering

Electrospinning has been recognized as an efficient and well-established technique capable of producing nanofibers by electrically charging a suspended droplet of polymer melt or solution.[25–32] Various polymers, including synthetic ones such as poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-*co*-glycolic acid) (PLGA), polystyrene, polyurethane (PU), polyethylene terephthalate (PET), and poly(L-lactic acid)-*co*-poly(ε-caprolactone) (PLACL), and biological materials, such as collagen, gelatin, and chitosan, have been successfully electrospun to obtain fibers with diameters ranging from 3 nm to 5 μm. Different parameters control the electrospinning process, including the solution properties, applied voltage, solution flow rate, humidity, and temperature. Using a simple and inexpensive setup, this technique not only provides an opportunity for control over the thickness and composition of nanofibers but also controls fiber diameter and porosity of the electrospun nanofiber meshes. Typically, nanofibers are collected as random, and aligned nanofibers with improved mechanical stability and degradation properties are also produced for specific applications. Whereas deposition of nanofibers on a static plate produces randomly oriented nanofibrous (100–650 nm) scaffolds, aligned nanofiber (250–650 nm) mats are fabricated using a rotating cylinder or disk collector with a sharp edge as shown in Fig. 5.2a and b. Coaxial electrospinning is a modification or extension of the traditional electrospinning technique with a major difference being a compound spinneret used. Using the spinneret, two components are fed through different coaxial capillary channels and are integrated into core-shell structured composite fibers to fulfill different application purposes. For example, bioactive composite scaffolds are fabricated using collagen (imparting bioactivity) as the shell and PCL (synthetic polymer) as the core (Fig. 5.2c).

Core-shell structured nanofibers (360–400 nm) prepared by coaxial electrospinning, have the advantages of being able to control the shell thickness and manipulate overall mechanical strength and degradation properties of the resulting composite nanofibers without changing their biocompatibility. Alternatively, core-shell structured composite nanofibers are functionalized for potential use in drug or growth factor encapsulation and release and development of highly sensitive sensors and tissue engineering applications. Tissue engineering is the application of knowledge and expertise from a multidisciplinary field to develop and manufacture therapeutic products that use the combination of matrix scaffolds with viable human cell systems or cell-responsive biomolecules derived from such cells for the repair, restoration, or regeneration of cells or tissue damaged by injury, disease, or congenital defects.

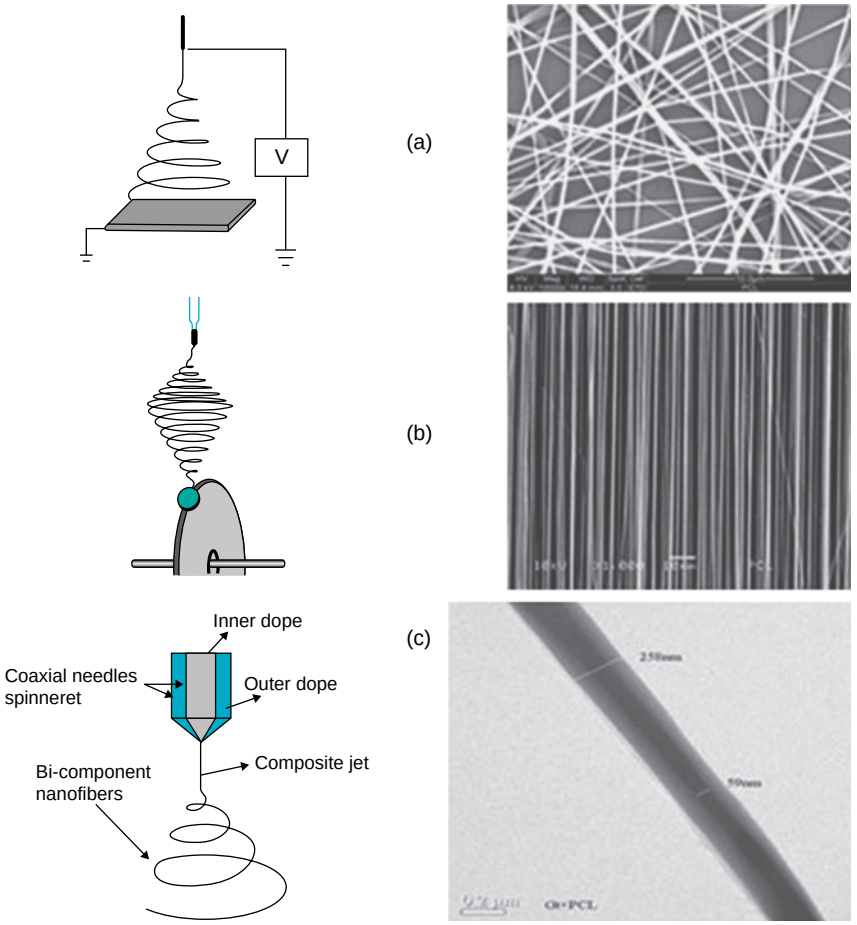

FIGURE 5.2 Schematics of electrospinning. (a) Random nanofibers produced by static collector. (b) Aligned nanofibers produced on a disk collector in a rotating wheel. (c) Coaxial electrospinning model for producing core-shell nanofibers.

Tissue engineering involves scaffolds or matrices to provide support for cells in order to express new ECM. The biocompatibility of scaffold materials actively participates in the signaling process for the requirement of safe degradation and provides a substratum for cell migration into the defect sites of the tissue. Potential applications of electrospun nanofibers for stem cell differentiation are envisioned in the fields of skin, bone, cartilage, blood vessels, cardiovascular diseases, nerves, and soft tissues.

5.1.2 Electrospun Nanoparticle Incorporated Natural Polymeric Scaffolds

5.1.2.1 Collagen Collagen is a fibrous protein found in animals, especially in the flesh and connective tissues of mammals. It is the most abundant protein in

mammals, constituting up to 35% of the whole-body protein content commonly created by fibroblast cells. Collagen is mostly found as elongated fibrils in fibrous tissues such as tendons, ligaments, and skin and is also abundant in corneas, cartilage, bone, blood vessels, the gut, and intervertebral discs. Collagen is a major ECM component that possesses a fibrous structure with fibrils of varying diameters (50–500 nm). This fibrils influence cell behavior by allowing cell attachment to the nanofeatured collagen matrix. Cells seeded on this nanofibrous matrix tend to maintain their normal phenotype and guided growth along the fiber orientation.

The motif behind the biomimetic nanostrategies is to dictate, control, and fabricate the morphology and composition of developed biomaterials. Nanoparticles are incorporated into natural or synthetic polymers to create functional polymeric composites suitable for tissue regeneration. Inorganic hydroxyl apatite (HAp) is being dispersed with preferential orientation so as to enhance bone tissue regeneration. HAp has inorganic crystalline nature same as that of natural bone and is biocompatible, bioactive, and osteoconductive in nature. Collagen and HAp biocomposite is a native ECM mimic and has the potential of replacing diseased skeletal bones. Because of potential biomedical applications, many studies report on the fabrication of bone-mimicking biocomposites of HAp and bioactive organic components such as collagen, gelatin, chondroitin sulfate, chitosan, and amphiphilic peptide.[33–36]

High levels of type I collagen and several noncollagenous proteins (e.g., osteopontin, bone sialoprotein, osteocalcin) constitute bone tissue. Collagen scaffolds get easily biodegraded and resorbed by the body and facilitate excellent attachment to cells. However, their mechanical properties are relatively low ($E \sim 100$ MPa), especially with respect to bone ($E \sim 2$–5 GPa),[37] and they are therefore highly cross-linked or found in composites, such as collagen–glycosaminoglycans for skin regeneration[38] or collagen–HAp for bone remodeling.[39] The advantage of the collagen and HAp devices in comparison with the synthetic PLGA devices[40] is that the biocomposite significantly inhibit the growth of bacterial pathogens, which is often associated with prosthesis. Although electrostatic co-spinning of nano HAp, and collagen improved the mechanical properties of the scaffold, much has to be done to exactly mimic the complex native nanostructured architecture of the bone. Collagen supported cell adhesion and proliferation, and HAp acted as a seed for biomineralization of osteoblasts in bone tissue regeneration.[41] The biocomposite of collagen and nanoHAp[42,43] is bioactive, osteoconductive, and osteoinductive and is a natural choice for bone grafting because it mimics the bone components. Bonelike orientation of c-axes of HAp nanocrystals with regular alignment along collagen fibrils are also fabricated.[44] The collagen–HAp composite, designed to simulate bone tissue, is produced using atelocollagen to reduce antigenicity by condensing $Ca(OH)_2/H_3PO_4$ suspension.[45]

Thus, electrospun nanofibrous collagen provides a native bonelike environment in the presence nanocrystalline HAp, enhancing regeneration of bone tissue or differentiation of stem cells into bone tissue.

5.1.2.2 Gelatin Gelatin is a protein obtained from the partial hydrolysis of collagen extracted from skin, bone, cartilage, ligaments, and so on. Gelatin is used as an alternative source of collagen to design tissue engineering scaffolds, mainly because of the lack of availability and high cost of collagen. Composite scaffolds of gelatin with other biodegradable synthetic polymers have been well adopted by many researchers. Moreover, these composite scaffolds with excellent biocompatibility, improved mechanical, and physical and chemical properties overcome the obstacles associated with the use of single natural polymers.[46] Interaction between cells and the scaffold material depends on various physicochemical properties of the material and particle size and surface properties that include topography, roughness, surface energy, and wettability.

Three-dimensional nanofiber-gelatin–apatite composite scaffolds were fabricated by Liu et al.[47] to mimic both the nanoscale native architecture and chemical composition of natural bone ECM. With a new thermally induced phase separation and porogen-leaching technique, these 3D nanofibrous gelatin scaffolds with well-defined macropores were designed. The inorganic HAp deposited all along the 3D porous structure is ideal for controlling surface topography and chemistry within complex nanostructures. And it was shown that these scaffolds have excellent biocompatibility and mechanical properties with enhanced osteoblast adhesion, proliferation, and differentiation suitable for bone tissue engineering.

5.1.2.3 Silk Fibroin Silk fibroin is considered as the most promising natural fibrous protein replacement for collagen in bone tissue engineering because of its biocompatibility, slow biodegradation, and excellent mechanical properties. In the past few years, two natural silk sources (e.g., silkworm silk *Bombyx mori* and spider dragline silk *Nephila clavipes*) have been processed for making nanofibers via electrospinning.[48–51] To improve the electrospinnability of silk protein solutions and to avoid potential influences of hazardous organic solvents such as hexafluoroisopropanol,[48] hexafluoroacetone,[49] and formic acid[51] toward the biocompatibility of the scaffolds, an all-aqueous electrospinning was attempted by Jin et al.[50] by blending silk fibroin with PEO at a ratio from 1 : 4 to 2 : 3. Methanol treatment of the electrospun scaffold renders water insolubility of the scaffold because of the structural conformational change into native β-sheet structure. Silk-based biocomposite nanofibers of HAp and bone morphogenetic protein 2 (BMP-2) were fabricated by Li et al.,[52] and an enhanced bone formation was observed by culturing with human bone marrow-derived mesenchymal stem cells (hMSCs). It was observed that the inclusion of BMP-2 and HAp with electrospun silk fibroin nanofibers resulted in the highest calcium deposition and upregulation of BMP-2 transcript levels compared with other electrospun silk-based scaffolds.

5.1.2.4 Chitosan Chitosan, an amino polysaccharide derived from the structural biopolymer chitin exists abundantly in crustacean shells (e.g., crabs) and plays a key role as that of collagen in higher vertebrates. Chitosan retains a number of salient features such as structural similarity to glycosaminoglycan found in bone, osteoconductivity, excellent biocompatibility, tailorable biodegradability,

low immunogenicity, and better mechanical properties[53–55] and at low cost. However, it is poorly electrospinnable and forms aggregates with non-electrospinnable HAp nanoparticles. Therefore, formulating a robust chitosan solution to generate nanofibrous HAp–chitosan biocomposite scaffolds is difficult. Because of these limitations in electrospinning of chitosan,[56,57] there are only a few reports on nanofibrous hydroxyapatite (HA)–chitosan composites for bone tissue engineering. Using ultrahigh-molecular-weight poly(ethylene oxide) (UHMWPEO) as a support polymer, Zhang et al.[58] fabricated composite chitosan nanofibers by a modified two-step approach.[59] In short, an *in situ* co-precipitation synthesis route was designed to overcome the problem of nanoparticles agglomeration and electrospinning process was carried out for the preparation of HAp–chitosan nanocomposite nanofibers with a higher (30 wt%) loading of HAp nanoparticles. It was confirmed with electron diffraction and X-ray diffraction analysis that the acetic acid used for chitosan dissolution had minor or no influence on the crystallinity of HAp nanoparticle incorporated within the nanocomposite nanofibrous structure. Bone regeneration ability of the scaffold was assessed on these HAp–chitosan nanocomposite nanofibrous scaffolds, and the results confirmed that the scaffolds had significantly enhanced bone formation compared with the pure chitosan scaffold.

5.2 SYNTHETIC ENROUTES

Multiple procedures and method combinations are used for the successful fabrication of a nanofibrous construct for stem cell or tissue regeneration. The scaffold needs to be stable in culture media; hence, natural polymeric scaffolds have limitations in direct application, highlighting the need for cross-linking of the electrospun natural protein-based scaffolds, which makes it stable during incubation in culture media.

5.2.1 Chemistry of Cross-Linking

Cross-linking is the process of chemically joining two or more molecules by a covalent bond. Cross-linking of proteins or carbohydrates depends on the availability of particular chemical groups that are capable of reacting with the specific kinds of functional groups that exist in proteins.

Despite the complexity of protein or carbohydrate structure, four major functional groups constitute for the vast majority of cross-linking and chemical modifications:

1. *Primary Amine Functionality ($-NH_2$):* The amine group exists at the N-terminus of each polypeptide chain and in the side chain of some amino acid residues.
2. *Carboxyl Groups (–COOH):* The carboxylic acid group exists at the C-terminus of each polypeptide chain and in the side chains of some amino acid residues.

3. *Sulfhydryl Functional Group (–SH):* The thiol group often helps in disulfide bond formation and exists in the side chain of cysteine amino acid.
4. *Carbonyl Functional Group (–CHO):* The aldehyde groups, which are often associated with carbohydrates and glycoproteins, are formed by oxidation.

When interconnected via the cross-linkers, these residues become stable toward degradation with improved mechanical strength. Glutaraldehyde solutions or vapors have been commonly used to cross-link protein-based and amino group containing carbohydrate scaffolds. The glutaraldehyde cross-linking technique is not expensive but efficiently cross-links over a variety of distances and reacts with many of the amino groups. The extent of cross-linking in an electrospun scaffold is directly proportional to the percentage of glutaraldehyde present in the cross-linking solution. The degree of nanofiber scaffold cross-linking increases as the percentage of glutaraldehyde present in the electrospinning solution increases but attains a maximum point where further no cross-linking can occur. However, some cytotoxity and calcification issues are associated with the glutaraldehyde cross-linker. Other methods for nanofibrous scaffold cross-linking are carbodiimide–ethanol techniques. Carbodiimide is a zero-length cross-linker with nominal potential cytotoxity issues and can be used to modulate material properties similar to glutaraldehyde. Genipin is a natural material cross-linker as a substitute for gluteraldehyde, carbodiimide, and isocyanate cross-linkers because of the cytotoxicity associated with these materials. Chitosan cross-links with ring-opening polymerization of a genipin double bond and the nucleophilic attack of chitosan on genipin. Despite its less cytotoxicity compared with other cross-linkers, there are only a few reports available with genipin cross-linking because of its high cost. Thus, there exists a demand for a new effective, nontoxic, economic cross-linker.

5.2.2 Elastomeric Scaffolds

An elastomer is a polymer that is elastic in nature and it has a relatively low Young's modulus and high yield strain compared with other synthetic and natural polymers. Elastin constitutes the natural elastomeric material present in various tissues of the human body. Many well-known elastomeric polymers, such as PU and biodegradable polyester urethane urea (PEUU), have been tried as cardiac patches, are biodegradable (poly(glycerol sebacate) [PGS]), and so on. Fong and Reneker[60] have electrospun styrene–butadiene–styrene triblock copolymer so as to fabricate elastomeric nanofibers with 100 nm diameters. Artelon (polyurethane urea elastomer) was electrospun[61] to obtain degradable nanofibers with an average diameter of 750 nm, and the biocompatibility studies were carried out using human fibroblasts. Stankus et al.[62] have electrospun biodegradable PEUU nanofiber scaffolds with tensile strengths ranging from 2.0 to 6.5 MPa and breaking strains from 850 to 1700% depending on the material axis, especially for regeneration of smooth muscle cells (Fig. 5.3). PGS, a tough biodegradable elastomer,[63] is being used in soft tissue engineering. PGS–gelatin nanofibrous scaffolds were fabricated by electrospinning

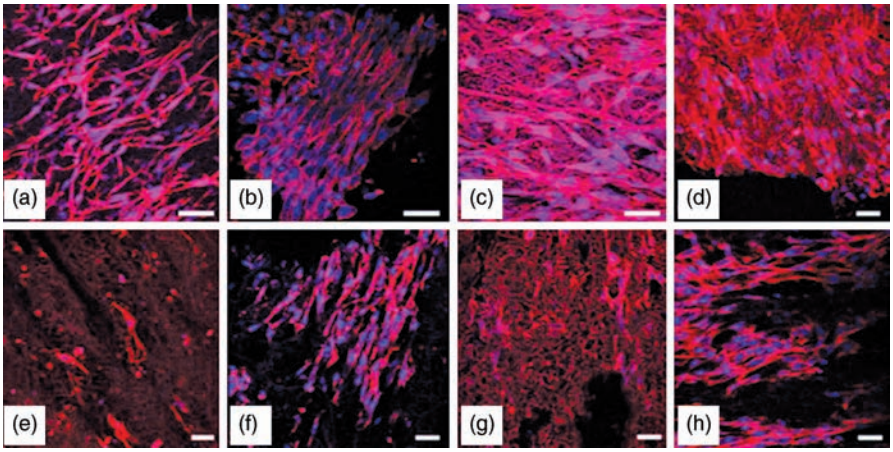

FIGURE 5.3 Fluorescent micrographs of SMC microintegrated e-PEUU constructs after 1 day of static culture (a), day 4 of perfusion culture (b), day 4 of perfusion culture (c), day 7 of perfusion culture (d), day 4 of static culture (e), high cell number surface image of day 4 of static culture (f), day 7 of static culture (g), and high cell number surface image of day 7 of static culture (h). Scale bar = 40 μm, red = f-actin and e-PEUU, blue = nuclei. Reproduced with copyright permission from Ref. [62].

to produce a unique ECM-like topography and were suggested as a potential biomaterial for myocardial infarction.[64]

5.2.3 pH Responsive Polymers

pH-sensitive or -responsive polymers are materials that respond to the changes in the pH of its surrounding medium. These polymers swell or collapse depending on the pH; this behaviour is exhibited because of the presence of acidic or basic functionality in the polymer chain. For example, whereas polyacrylic acid (PAA) is acidic in nature but swells at basic pH, chitosan with its basic amino groups swells if acidic changes occur in its surroundings. This pH-mediated response of the polymers is useful for the release of drug molecules or growth factors encapsulated within these polymers. Therefore, the application of these polymers will be enormous so as to work under physiological pH conditions. The phenomenon behind the swelling behaviour is the volume transition associated with the ionized state of the polyelectrolyte from the neutral state. Thus, all acidic polymers are base sensitive (polymethacrylic acid, xylan, etc.) and basic polymers (PEI, poly aniline, etc.) are acid sensitive. Amonodisperse triblock copolymer of poly(methyl methacrylate)-*block*-poly[2-(diethylamino)ethyl methacrylate]-*block*-poly(methyl methacrylate) ($PMMA_{273}$-*b*-$PDEA_{688}$-*b*-$PMMA_{273}$) was synthesized[65] via group transfer polymerization as a pH-responsive system and electrospun 35% of the copolymer to obtain the pH-sensitive scaffold. Wang et al.[66] fabricated electrospun pH-responsive γ-PGA

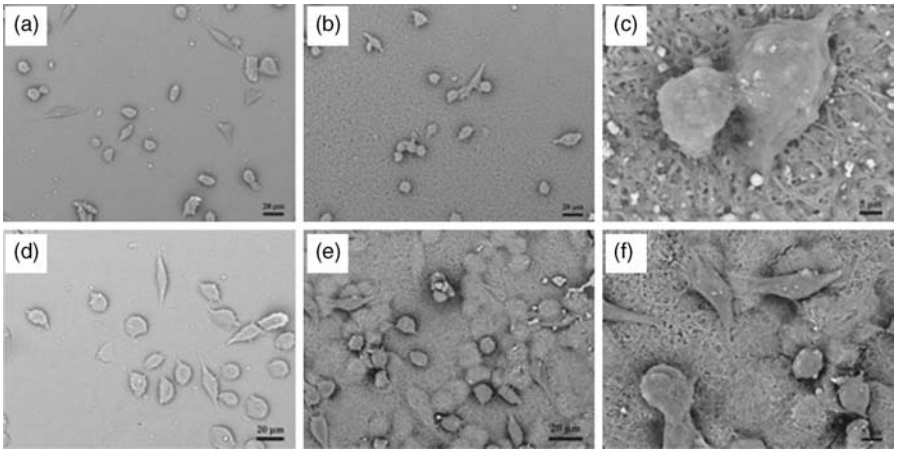

FIGURE 5.4 SEM micrographs of fibroblasts attached onto cover slips (a) and electrospun γ-PGA nanofibers formed using 5 wt% TFA as a solvent (b), respectively, after 8 h of culture. (c) High magnification image of (b). (d and e) SEM micrographs of fibroblasts proliferated onto cover slips and electrospun γ-PGA nanofibers formed using 5 wt% TFA as a solvent, respectively, after 3 days of culture. (f) High magnification image of (e). Reproduced with copyright permission from Ref. [66].

nanofibers that have an excellent biocompatibility to promote the cell adhesion and proliferation (Fig. 5.4).

5.2.4 Thermo-Responsive Polymer Fabrication and Engineering

Thermo- or temperature-responsive polymers respond to temperature change by expansion of dimension or size. This character of these polymers is used for the release of drug molecules incorporated or encapsulated drug from within the polymer. Poly(*N*-isopropylacrylamide) (PNIPAm) is a temperature-responsive polymer that can be synthesized from NIPAm monomer. It can be made to a 3D hydrogel architecture when cross-linked with *N,N′*-methylene-bis-acrylamide (MBAm) or *′-N, N′*-cystamine-bis-acrylamide (CBAm). In the presence of water when heated above 32 °C, it undergoes a swollen hydrated state to a shrunken dehydrated state by a reversible lower critical solution temperature phase transition, losing about 90% of its mass, by expelling its liquid contents at human body temperature. Thus, the polymer is useful for tissue engineering applications and in drug delivery. Azarbayjani et al.[67] have electrospun a series of nanofibrous membranes from poly(vinyl alcohol) (PVA) and PNIPAm blends to develop a sustained topical delivery of levothyroxine (T_4). These nanofiber mats were suggested as promising carriers for keeping the drugs concentrated on the skin over a prolonged period with reduced systemic uptake. Similar applications of PNIPAm in tissue engineering are well known, but the applications of PNIPAm nanofibers with tissue engineering applications is yet to be extensively studied.

5.2.5 Modified Electrospinning Processes

5.2.5.1 Simultaneous Electrospinning and Electrospraying HAp nanoparticles were electrosprayed on PLACL–gelatin nanofibers to produce PLACL–gelatin–HAp scaffolds with controlled morphology for application in bone tissue engineering. Gupta et al.[68] used a simultaneous electrospraying and electrospinning (Fig. 5.5) concept and fabricated PLACL–gelatin–HAp nanofibers and compared their mechanical and cellular properties with blend electrospun PLACL–gelatin–HAp scaffolds.

Electrospun PLACL–gelatin–HAp (blend) nanofibers had a drawback of trapping HAp inside the nanofibers (diameter, 198 ± 107 nm), but the HAp nanoparticles were found uniformly sprayed forming a layer of HA on the surface of the other PLACL–gelatin–HAp scaffold (diameter, 406 ± 155 nm). The tensile stress for HAp electrosprayed scaffold was higher than PLACL–gelatin–HAp (blend) scaffold because the electrospraying of HAp nanoparticles resulted in superficial dispersion of HAp nanoparticles. A significant increase in hFOB proliferation was observed on the HAp electrosprayed scaffold compared with the PLACL–gelatin–HAp (blend) nanofibers after 15 days of cell seeding. Furthermore, the electrosprayed scaffolds showed 50% higher biomineralization than the PLACL–gelatin–HAp (blend), thus proving the versatility of the electrospraying method compared with the blend technique with respect to scaffold design for bone tissue engineering. Jayasinghe and coworkers[69,70] have electrosprayed jurkat cells and assessed for their viability by

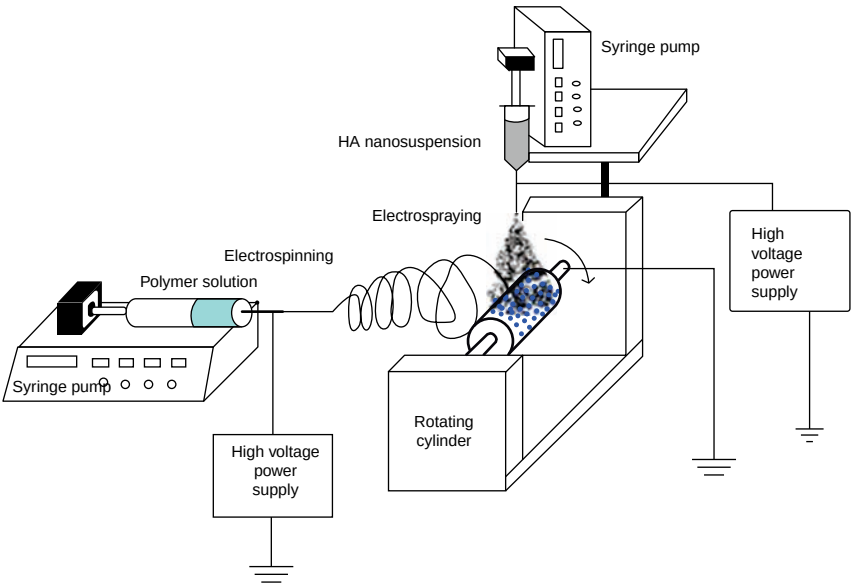

FIGURE 5.5 Schematic representation of simultaneous electrospraying and electrospinning.

way of trypan blue staining. This methodology of bioelectrospraying[71] is said to have a wide range of applications spanning from bio-analytics and diagnostics to the possible creation of synthetic tissues for repairing and replacing damaged or aging tissues to the targeted and controlled delivery of personalized medicine through experimental or medical cells or genes.

5.2.5.2 Coaxial Electrospinning Coaxial electrospinning is a method of electrospinning in which the core polymer is encapsulated by another polymer that forms the shell of the electrospun nanofibers because of electrostatic voltage applied via the shell polymer. In this method, only the shell polymer is electrospun in principle; the core polymer is just dragged inside the shell, and in most cases, it is a nonspinnable material polymer. In a tissue or stem cell regeneration perspective, it is a novel method for the development of controlled release of encapsulated growth factor or related differentiating material for the stem cells. Sahoo et al.[72] have shown the growth factor delivery via core shell nanofibers with PLGA as shell material and basic fibroblast growth factor (bFGF) as core. They realized that the material ensures sustained release of growth factors up to 2 weeks. They observed the nanofibrous scaffold enhanced cell attachment, proliferation, and fibroblastic differentiation of bone marrow stem cells, which they further confirmed with increased collagen production and upregulated gene expression of specific ECM proteins. Su et al.[73] have demonstrated the controlled release of BMP-2 and dexamethasone using core–shell PLLACL–collagen nanofibers for bone tissue engineering applications (Fig. 5.6). Similar approach with respect to stem cell differentiation is yet to be extensively explored.

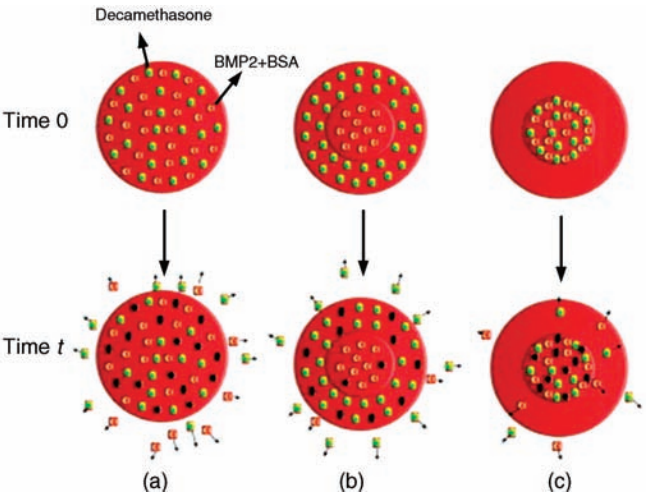

FIGURE 5.6 Schematic representation of the release of DEX and BMP2 from electrospun nanofibers (a, b, and c). Reproduced with copyright permission from Ref. [73].

5.3 NOVEL NANOFIBROUS STRATEGIES FOR STEM CELL REGENERATION AND DIFFERENTIATION

Stem cells are biological cells found in all multicellular organisms and have the capacity to self-renew; they divide via mitotic cell division and differentiate into diverse specialized cell types (tissue or organ). In mammals, there are two broad types of stem cells, embryonic stem cells, which are isolated from the inner cell mass of blastocysts, and adult stem cells, which are found in various tissues. During development of an embryo, stem cells differentiate into many different types of specialized cells, and they also maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues. Another type is adult stem cells, which are undifferentiated cells found along with the differentiated cells in an organ or tissue, which can renew themselves and can differentiate to yield major specialized cell types of organ or tissue.

In mature organisms, stem cells and progenitor cells act as a repair system in the body, replenishing matured tissues. These adult stem cells maintain and repair the tissues in which they constitute. They can be collected from tissues such as adipose tissue, bone marrow, mammary tissue, central nervous system, olfactory bulb, and so on. Transdifferentiation ability has also been demonstrated by adult stem cells (i.e., they can switch their specific developmental lineage to another cell type of a different lineage).[74] However, the molecular mechanism that drives transdifferentiation is not clearly understood. Stem cells have the unique property of self-renewal without differentiation if appropriate biological and physical induction conditions are provided. In the context of tissue engineering, the use of stem cells has the following advantages compared with engineered tissue constructs: (1) they have high proliferative capacity, (2) they provide excellent regenerative capability that will likely lead to desired integrity and functionality of the engineered construct, (3) they make it possible to contemplate multifunctional tissue constructs (e.g., osteochondral tissue), and (4) they reduce or eliminate tissue rejection or failure.

Although the application of living cell therapy is associated with challenges, stem cells constitute the functional elements of tissue engineering and regenerative medicine.[75] The following are the prerequisites for researchers and clinicians to work out the success in cell-based treatments. For transplantation practices, stem cells must be reproducibly made to (1) differentiate into the desired cell types; (2) survive in the recipient after transplantation; (3) integrate into the surrounding tissue after transplantation; (4) function appropriately for the duration of the recipient's life; and (5) avoid harming the recipient in any way.

Researchers are working in the direction of minimizing or avoiding the problem of immune rejection of regenerated tissues with different research strategies. The most commonly studied stem cells are the bone marrow stem cells, especially the MSCs and hematopoietic stem cells (HSCs). Under controlled conditions, the MSCs have the ability to differentiate into cell lineages[76] such as osteoblasts, chondrocytes, cardiomyocytes, and fibroblasts. The *in vitro* cell culture of hMSCs, proliferation and differentiation into tissue specific cell phenotype such as

chondrogenic, osteogenic, adipogenic, and myogenic cells with the application of a biological or physical stimuli, is well understood and established.[77–80] The hMSCs have enormous therapeutic potential for treatment of damaged or diseased tissue; the complexity of events associated with such transformation of these precursor cells leaves many unanswered questions about morphologic, structural, proteomic, and functional changes in stem cells. Thus, there exist a need for better understanding of hMSC behavior that would allow more effective approaches to cell expansion *in vitro* and differentiation to a specific phenotype. Hence, there is a need for favorable scaffolds and engineering for hMSCs to orient, adhere, proliferate, and differentiate.

The multilineage differentiation potential of MSCs on 3D PCL nanofibrous scaffolds was demonstrated by Li et al.[81] They tested the ability of the scaffold to support and maintain multilineage differentiation of bone marrow-derived hMSCs *in vitro* by culturing in different differentiation media such as adipogenic, chondrogenic, or osteogenic and found the PCL scaffold as the promising one. The differentiation potential of MSCs into hepatocytes was observed by Kazemnejad et al.[82] on PCL–collagen–polyethersulfone scaffolds. The ability of the differentiated hepatocyte cells to produce albumin, urea, serum glutamic, pyruvic, transaminase, and serum oxaloacetate aminotransferase on the scaffolds further confirms the supporting role of the nanofibrous scaffolds. The osteoblastic differentiation potential of MSCs on poly(L-lactic acid) (PLLA)–collagen nanofibers was demonstrated by Schofer et al., who identified the advantages together with disadvantages of more stable PLLA–collagen fibers with respect to osteoblastic differentiation.[83] *In vitro* differentiation of MSCs into cardiac cells is commonly carried being out by exposure to 5-azacytidine, a DNA demethylating agent.[84] Expression of many cardiac specific genes and peptides was observed.[85] Recently, Nerurkar et al.[86] observed improved cellular ingress into electrospun scaffolds by adopting dynamic culture of MSCs on aligned PCL nanofibrous scaffolds. This dynamic culture modification for MSC culture has increased cellular infiltration and facilitated the use of aligned electrospun scaffolds for tissue engineering. In our laboratories, we studied the neuronal differentiation potential of hMSCs on PLCL–collagen scaffolds. The results of our study showed the neuronal phenotype of MSC differentiated cells together with the expression of nerve proteins such as NF200 and nestin.[87] Thus, with a better understanding of the behavior of MSCs on electrospun nanofiber scaffolds, a "stem cell–scaffold construct" might find real application in regenerative medicine curing various human diseases.

The transplantation of embryonic stem cells (ESCs) for the treatment of peripheral nerve injuries and possibly spinal cord injuries has also been demonstrated.[88,89] Functionalized electrospun nanofibrous scaffold with growth factors was found to enhance the differentiation of ESCs into neurons and oligodendrocytes.[90] Xie et al.[91] demonstrated that the ESCs are differentiated into neural cell lineages guided by electrospun nanofibrous scaffolds. They also found the ESCs to promote and direct neurite outgrowth. The novel strategy of using a combination of electrospun scaffolds together with ESC-derived neural progenitor cells might lead to better nerve repair. Lam et al.[92] immobilized bFGF or epidermal growth factor (EGF) onto

aligned PLLA nanofibers using heparin as the adapter molecule and elucidated the effect of growth factors on ESC differentiation into neural cells with significant promotion of axonal growth. Immobilization of bFGF and EGF in aligned nanofibers was successfully carried out by these researchers to promote neural tissue regeneration. Nuria and Carlos[93] proposed that 3D cell culture on self-assembling peptide nanofibrous scaffold could provide a unique microenvironment permissive to promote the differentiation of mouse ESCs into osteoblast-like cells while maintaining their own regenerative capacity. Kamal et al.[94] fabricated 3D polyamide fibrillar surfaces for the self-renewal of mouse ESCs through mechanism involving Rac and P13K/AKT signaling, thus exhibiting the role of nanostructural scaffold morphology for ESC proliferation. Optimization of a suitable nanostructure or microenvironment is the requirement for efficient differentiation of ESCs in 3D scaffold structures further led to the research on scaffold pore size, increasing mechanical stiffness, increasing the cell seeding density, co-culturing with stromal cells,[95,96] and so on. Hashemi et al.[97] have demonstrated very recently the promotion of stemness and pluripotency (Fig. 5.7) with collagen-grafted polyethersulfone (PES) 3D nanofibrous scaffold culturing mESCs.

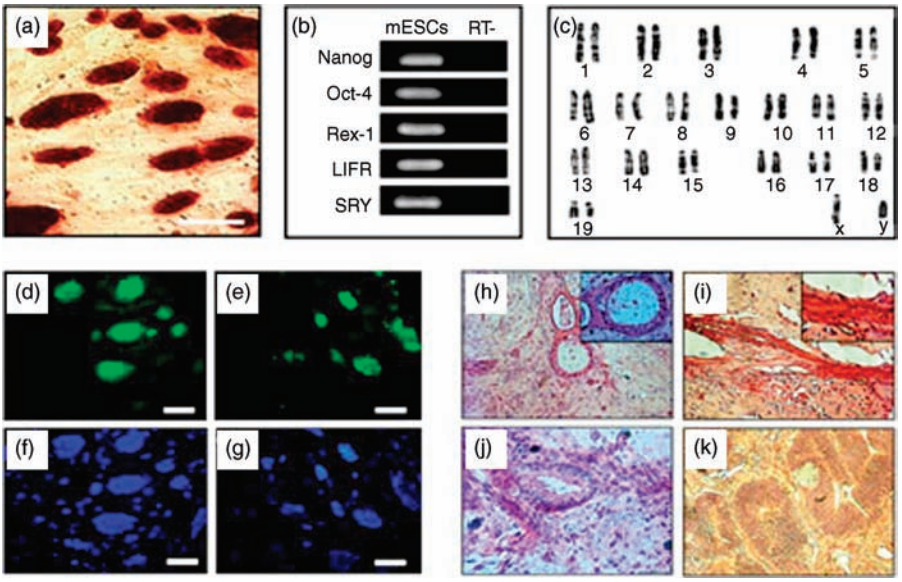

FIGURE 5.7 Characterization of the mESCs cultured on MEF in the presence of LIF after 10 passages: alkaline phosphatase assay (a); RT-PCR analysis of expression of embryonic stem cell–specific genes (b); Giemsa-banded karyotype of an embryonic stem cell showing a normal 40 XY karyotype (c); immunofluorescence staining of OCT-4 (d, f) and SSEA-1 (e, g); histologic analysis of teratoma-derived from mESCs, gutlike structures, muscle cells, secretory epithelium, and neural rosettes (h–k). Reproduced with copyright permission from Ref. [97].

Human umbilical cord blood (UCB) stem cells[98] are an alternative source of hematopoietic precursors for allogeneic stem cell transplantation in children with inborn errors or malignant diseases. HSCs, originating from bone marrow, are used for the treatment of many bloodborne and other diseases, including sickle cell anemia, thalassemia, aplastic anemia, leukemia, metabolic disorders, and certain genetic immunodeficiencies.[99] The cord blood stem cells show a higher proliferative capacity and expansion potential. Allogeneic stem cell transplantation is limited because of the lack of suitable bone marrow donors and the risk of graft-versus-host diseases. The percentage of stem cells is higher in cord blood than in the bone marrow, and the main merits of UCB stem cells over the other stem cell sources are (1) easy to recover, (2) no health risks for the mother or newborn, (3) immediate disposition at the cryobank, (4) low incidence of rejection of the transplant, (5) high cellular plasticity, (6) low possibilities of transmission of viral diseases, (7) low cost of the procedure, and (8) easy possibilities to create cord blood banks so as to store samples.

Transplantation protocols into adults is limited because of the low number of progenitors in cord blood harvest and due to this, expanding HSCs *ex vivo* to get sufficient number of cells for transplantation became a need. Several studies have demonstrated[100] the application of nanofibrous scaffolds for enhancement of cellular responses such as cell adhesion and cell phenotype maintenance. Researches on the influence of nanotopographical cues and biochemical cues on the nanofiber surface and their synergistic influence toward HSC adhesion, proliferation and phenotypic maintenance are also established. The highest expansion efficiency of CD34+, CD45+ cells, and colony-forming unit potential was observed in surface-aminated electrospun nanofibrous scaffolds compared with the unmodified, surface-hydroxylated, surface-carboxylated[101] nanofibrous scaffolds. Amino groups were conjugated as spacers to nanofiber surfaces, and it was found[102] that the cell–substratum interaction dictated the HSC–progenitor cell proliferation and self-renewal in cytokine supplemented expansion. Aminated nanofiber scaffolds and PCL–collagen nanofiber scaffolds were found to enhance the HSC–substrate adhesion and proliferation of progenitor cells. This formed the basis for research on specific cell adhesion molecules such as fibronectin in combination with the nanofiber substratum toward HSC adhesion and expansion *ex vivo* to solve various diseases.

Unrestricted somatic stem cells (USSCs) were seeded on electrospun PES nanofiber mats with plasma treatment and collagen grafting, and their biocompatibility and application in tissue engineering was investigated. Imam et al.[103] observed the infiltration of stem cells into the collagen grafted nanofibers after 7 days of cell culture, thus making collagen-grafted PES nanofibers an ideal candidate to form 3D structures in tissue engineering. They further observed that the PES–collagen nanofibers[104] have the highest capacity to support osteogenic differentiation and infiltration of stem cells into the 3D nanostructure, which they confirmed via assessment of osteogenic markers and histologic examination. Results from their study concluded that the PES–collagen scaffolds could act as a potential 3D bone graft with capacity for bone healing and regeneration *in vivo*.

5.4 CONCLUSIONS

Scaffolds that mimic the natural ECM are considered the most ideal scaffolds for tissue or stem cell regeneration. Bioengineers aim for the development of suitable substrates for tissue regeneration using various and ultimate nano- or microtechnologies. To find a synthetic solution to the natural scaffold materials, many groups use nanofibrous scaffold comprising various novel features such as cross-linking, surface modification, growth factor inclusion or sustained release, drug or antioxidant inclusion, and nanostructural modifications in fiber alignment. We have identified and provided in this chapter the summary of the above-stated research works so as to provide an overall outlook. Designing an optimized biomimetic ECM scaffold is an achievable task with better understanding of the chemistry of the scaffold and its structure and pattern along with the biochemical signals associated with stem cell differentiation and proliferation.

ACKNOWLEDGMENT

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6

INTEGRATING TOP-DOWN AND BOTTOM-UP SCAFFOLDING TISSUE ENGINEERING APPROACH FOR BONE REGENERATION

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6.1 INTRODUCTION

Tissue engineering (TE) as an interdisciplinary field of research aims at restoring, maintaining, or improving tissue function through applying the principles of biology, medicine, and engineering science.[1] Since its emergence in the 1980s, the field of TE in conjunction with regenerative medicine has been continuing to evolve, for example, through wound healing,[2,3] skin tissue engineering,[4–6] nerve regeneration,[7,8] cardiovascular tissue engineering,[9] bone and cartilage tissue engineering,[10] and others.[11,12]

Cells, scaffolds, and growth-stimulating bioactive factors are generally referred to as the three key components of engineered tissues in TE.[1] A common strategy in TE is combining cells, biodegradable scaffolds, and bioactive factors to replicate natural processes of tissue regeneration and development.[10] The interactions among these components are imperative to achieve biologically functional engineered tissue. In human tissue, cells are normally anchorage dependent, residing in an extracellular matrix (ECM). This ECM generally provides not only structural support and a physical environment but also bioactive cues and a reservoir of growth factors.[13] The

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synthetic scaffolds for an engineered tissue are regarded as a kind of ECM. However, ECM in native tissues possesses complex compositions and a dynamic nature, which bring multiple biological functions such as cell adhesion, migration, proliferation, and differentiation. Ideal scaffolds should therefore mimic the features of the native ECM of the target tissue. Nevertheless, the complexity of ECM makes it difficult to mimic exactly the structure and functions of native ECM in synthetic scaffolds.[14]

Therefore, the focus in tissue engineering is how to manipulate the process to integrate the key components of TE, trying to replicate the natural structure of tissue and mimic the functions of native ECM, at least partially. There are many technologies developed to achieve these aims. Although these techniques have succeeded in making biomimetic scaffolds, they have their own limitations. This chapter reviews the bone TE strategies involved in preparation of scaffolds and briefly discusses the drawbacks and advantages of these strategies.

6.2 CLINIC NEEDS IN BONE REGENERATION FIELDS

Every year, there are roughly 1 million bone grafting procedures in the United States and European Union.[15] These include indications arising from resection of primary and metastatic tumors, bone loss after skeletal trauma, failed fracture healing, spinal arthrodesis, and trabecular voids. In addition, more than 20 million people in the United States are totally edentulous.[16] About half a million children worldwide are born annually with congenital craniofacial deformities, such as cleft palate and hypertelorism.[17] Current treatments in clinic are based on autologous and allogeneic bone grafts.[18–22] Autografts have been the gold standard of bone replacement for many years because they provide the patient's own osteogenic cells, ECM, and essential osteoinductive factors needed for bone healing and regeneration.[21,23] Because an autograft is harvested from the patient's own body, there is a limited supply and morbidity of the harvest site, and the additional trauma is a concern. Although autograft is highly efficient for bone repair, the outcome for large bone defects is less predictable. Allografts could be used as an alternative for treating bone defects. However, allografts could introduce the possibilities of immune system rejection, pathogen disease transmission from donor to recipient, and infections after the transplantation.[24]

Therefore, biomaterials for bone defects, as an alternative to those two bone grafts, have been extensively studied to meet the increasing clinical demand. Currently, all kinds of biomaterials, including metals, ceramics, and polymers, have been studied for bone regeneration. However, none of these biomaterials, by themselves, can currently be used for full recovery of the patient. Metals exhibit poor integration with the tissue at the implantation site because of a lack of degradability, although they provide mechanical support at the site of the defect. Ceramics, because of their low tensile strength and brittleness, have limited application in loading-bearing sites. Polymers have been extensively used in drug delivery systems but have limitations in bone tissue engineering because of their low compressive strength and acid degradation products. It is clear that an adequate bone graft is yet to be found.

6.3 BONE REGENERATION STRATEGIES AND TECHNIQUES

Scaffolds need to mimic the natural structure of regenerated tissue to obtain optimal regeneration of biological functions. From a perspective of tissue engineering, cells, ECM, cell–matrix interactions, and bioactive factors should be involved to achieve the regenerated functions. For the components mentioned, an appropriate three-dimensional (3D) scaffold is an essential component for a tissue engineering strategy because scaffolds provide physical and mechanical support, spatial structure, and an adequate biochemical environment for cell behavior.[10] Scaffolds applied in TE need essential properties, including pore size, porosity, mechanical properties, and signal presentation.

Bone is a dynamic, highly vascularized tissue with hierarchic structure in a 3D configuration.[15] Therefore, the ideal scaffold should mimic the bone structure and provide a 3D microenvironment for growing new tissue in the scaffold. However, the coordination of all of these key components in an optimal spatial and time-dependent fashion will affect the ultimate results of regenerated tissues. There are many strategies or techniques for making bone constructs for tissue regeneration. From a fabrication perspective, these strategies can be generally implemented in two approaches: top down and bottom up.

6.3.1 Top-Down Tissue Engineering

6.3.1.1 Concept Since its emergence in the 1980s,[1,25] TE began to develop different approaches for tissue regeneration. The top-down approach represents the most traditional and typical one. Top-down tissue engineering approach generally uses well-defined porous scaffolds with tailored properties and signals as a template to induce desired cell response, leading to engineered tissues and organs. Specifically, to construct engineered bones, bone-forming cells or stem cells are seeded onto prefabricated porous scaffolds with controlled release of growth factors to induce bone formation. The essential properties of the scaffold include porosity, interconnectivity and pore size, mechanical strength, and biodegradability. Scaffolds as a template should possess similar functions to natural ECM. Scaffolds must possess a fully interconnected porous structure and open macropores for efficient nutrient and metabolite transport. The pores also facilitate the neovascularization of the construct from the surrounding tissue at the same time. However, the porosity will affect the mechanical properties that are required to balance the degradability of the scaffold. The mechanical properties of the implanted scaffold should ideally match those of living bone, so that no stress shielding or compression or deformation of the scaffold by the surrounding tissues takes place.[26–28] Therefore, the extent of porosity should be balanced with mechanical properties so that they both meet the demands of a specific regenerating tissue. To further enhance cellular adhesion and proliferation on the scaffold, the surface could be modified to be osteoconductive. Many different cell-interacting ligands, such as the RGD cell-adhesive ligand, could be grafted to the scaffold to provide biological cues for cell growth. The scaffolds may be used to load growth factors or to serve as a delivery vehicle or reservoir for

exogenous bioactive molecules to enhance regeneration. Many methods have been developed to produce scaffolds with adequate properties as mentioned earlier based on the top-down concept. An adequate processing technique should be performed on selected biodegradable materials. A description and discussion of these techniques is given in the following sections.

6.3.1.2 Processing Techniques Many techniques have been developed to prepare porous ceramic, polymer, or composite scaffolds. Gel casting of foams is an early developed technique for fabricating ceramic scaffolds with high mechanical strength.[29–31] This technique commonly results in a poorly interconnected pore structure and nonuniform pore size distribution;[32] however, these properties can be improved by using a sol–gel material and a gel-casting hybrid process.[33] The ceramic foam fabricated with this hybrid method exhibited sponge-like structures with uniform large pores and smaller pores distributed on the walls of the larger pores. The sizes of big and small pores were within 500–800 and 50–300 μm, respectively.[33]

Replication of a polymer sponge is a typical technique for producing ceramic scaffolds.[34,35] The replication method uses a sacrificial template (e.g., polyurethane foam) coated by a ceramic (or glass) slurry. After drying the ceramic slurry, the polymer template is slowly removed by thermal degradation, and the remaining ceramic is further sintered. The process replicates the macrostructure of the starting sacrificial polymer foam.[36–38] However, the low compressive strength of the scaffolds produced by this method limits their application in the repair of load-bearing bone defects.[39] Ramay and Zhang combined the gel-casting and polymer sponge methods to produce porous hydroxyapatite scaffolds with high mechanical properties.[32] A compressive modulus of 8 GPa and yield strength of 5 MPa for the scaffold with hydroxyapatite (HA) concentration of 50 wt% were achieved.[39] Fu et al. used a new method of direct-ink-write assembly of a hydrogel-based ink to fabricate bioactive glass scaffolds. Porous glass scaffolds with combined high compressive strength (136 MPa) and porosity (60%) were obtained,[40,41] which were comparable in mechanical properties to those of cortical bone and a porosity comparable to that of trabecular bone. The template-casting method is another technique that is used to produce porous ceramic scaffolds[41] and polymer scaffolds.[42–44] Recently, Yang and coworkers developed a template-casting technique to produce scaffolds with improved porous structure and mechanical strength. Scaffold composition and architecture were spatially regulated by controlling bead size and arrangement.[45–47]

For producing porous polymer scaffold, solvent casting and particulate leaching is the best known and most widely used method for the preparation of bone tissue engineering scaffolds because of its simple operation and adequate control of the pore size and porosity. After casting a dissolved polymer with a porogen, the solidified polymer is placed in a water bath to leach out the porogen, thus yielding an interconnected porous network. In this method, the particle size and amount of the porogen can be controlled. However, this technique is not applicable to ceramic scaffolds because the ceramic matrix obstructs complete removal of the porogen in the leaching step, resulting in a less interconnected network. Ever since Mikos et al. developed this technique to produce PLLA and PLGA polymer scaffolds,[48] many

researchers have used this technology to produce porous polymeric scaffolds.[49–53] There are many variations to the solvent casting and particulate leaching technique.[54] For example, any water-soluble porogen, different combinations of polymers and solvents, and varying compositions can be used in the casting step. The porogen can be also poured into a mold and partially fused using humidity to increase pore interconnectivity. PLLA and PLGA porous scaffolds have been produced with this modified method.[55–57] However, this method has some disadvantages; for example, the use of highly toxic solvents for polymer dissolution and the residual solvent remaining in the scaffold is a concern, and the residual porogen remaining in the polymer matrix after the leaching step can lead to enclosed and unconnected cavities.

In the thermally induced phase separation technique, a polymer such as PLLA, PLGA, or PCL is dissolved in an appropriate solvent (e.g., chloroform, dichloromethane) to obtain a homogeneous mixture. Next, the mixture is cooled below the solvent melting point to induce phase separation.[58] Then the mixture is quenched to form a two-phase solid, and the solvent is sublimated to yield a porous scaffold. The porosity and architecture of the polymer scaffold in this processing technique are generally affected by the cooling rate and melting temperature of the solvent.[59,60]

Freeze drying can also be used to fabricate scaffolds. An emulsified polymer solution is poured into a metal mold with the desired dimensions and allowed to freeze. Then the solvent is removed by freeze drying to yield a porous scaffold. However, the pores generated by this technique are relatively small.

Major concerns with typical solvent-casting strategies are the use of organic solvents and the toxicity of the residual solvent remaining in the scaffold after drying. A modified method is the gas-foaming technique, which does not require the addition of organic solvents. Compressed polymer disks in a mold are treated with high-pressure CO_2 or supercritical CO_2.[61–65] The nucleation in the polymer occurs when the pressure quickly decreases, thus forming pores. The pore size can be controlled by the reduction rate of pressure, but the pores produced by this technique are not interconnected. The combination of particulate leaching and gas foaming can improve the interconnectivity of the pores.[51,66]

Fiber bonding and electrospinning are fiber-fabricating technologies that create porous scaffolds composed of nano- and microscale biodegradable fibers. Many biocompatible polymers, such as PGA, PLGA, and PCL, are electrospun into porous nanofiber scaffolds with high porosities.[52,67]

Rapid prototyping by solid free-form technology (SFF) is used to produce porous scaffolds with well-defined pore geometry. This technology includes 3D printing,[68–70] laser sintering,[71–74] and stereolithography with variants.[75,76] Using computer-assisted design (CAD), this technique can produce fully interconnected porous scaffolds with well-defined pore geometry and complex pore architectures at the microscale. This technique has advantage over conventional fabrication techniques because the scaffold pore size and geometry can be designed electronically and mathematically. In a variant of this technique, a sacrificial wax mold is fabricated by an SFF technique such as fused deposition modeling (FDM). Then an *in situ* cross-linkable macromer is injected in the pore volume of the scaffold and allowed to cross-link by photo- or redox-initiated polymerization, rendering the polymer

insoluble in organic solvents. Then the infused mold is ether or wax, good solvent for ether, to leach out the wax, leaving behind a scaffold with well-defined pore geometry.[77] This technique can be used to fabricate porous uncross-linked or cross-linked polymer and hydrogel scaffolds with well-defined pore geometry. SFF technology has also been used to fabricate β-tricalcium phosphate (β-TCP)[78] and HA[79–81] scaffolds for bone regeneration. Toughness and strength in SFF scaffolds can be enhanced by adding a ceramic ink to the polymer phase.[82–85]

Other techniques for scaffold fabrication exist, such as melt molding and extrusion, which are not described here. These usually involve semi-industrial macrofabrication processes and extreme fabrication conditions, which are not compatible with the microscale environments for cells. However, the end product can be modified chemically after fabrication for cellular biocompatibility, although this can be more easily achieved by the previously described methods.

6.3.1.3 Limitations and Challenges The top-down approach using prefabricated scaffolds has a number of advantages. The materials used are diverse, ranging from ‘ceramics to polymers and hydrogels. These techniques can also produce porous scaffolds with high mechanical properties by altering the porosity and pore architecture. However, the top-down approach also has certain disadvantages. In this approach, the scaffold is expected to promote proliferation and differentiation of the cells seeded in the prefabricated biodegradable scaffold and create ECM. Although ceramic, polymer, and composite scaffolds fabricated by the top-down approach have been used as TE scaffolds, these porous biodegradable constructs often lack biological recognition cues. For example, they often lack osteoinductivity for bone tissue engineering. Postfabrication cell seeding into porous scaffolds is also inefficient because the ability of cells to penetrate the central part of the scaffold is limited, which leads to inhomogeneous distribution of cells in the scaffold and insufficient vasculature ingrowth. An ideal TE scaffold should mimic the native ECM and promote cell adhesion, growth, and differentiation.[86,87] To achieve this purpose and overcome drawbacks of the top-down approach, bioactive molecules, including growth factors, short peptides, and ECM proteins, are deposited, attached, or conjugated to the scaffold. For example, Jabbari and colleagues have shown that attached of a cell-adhesive RGD peptide and an osteoinductive peptide derived from bone morphogenetic protein 2 (BMP-2) synergistically enhances osteogenic differentiation of bone marrow stromal cells (bMSCs) and mineralized matrix formation.[88] Other efforts made to improve cell seeding include flow perfusion of the cell suspension inside the scaffold and using scaffolds with larger pore size.[89–91] However, despite these advances in surface engineering, biomimetic design, and conjugation methodologies to modify the scaffold microenvironment, top-down approaches still have difficulty recreating the intricate structure characters of tissues at micro- or nanoscale.

6.3.2 Modular Tissue Engineering (Bottom-Up Approach)

6.3.2.1 Concept The bottom-up approach aims to address the challenges of the top-down approach in mimicking the microstructural features of the tissue from the

opposite direction. The bottom-up approach builds a single unit at the micro- or nanoscale that serves as a building block for further assembly to a larger tissue scale. These modular units can be created in many different ways, such as cell sheeting, cell-laden microfabrication, or 3D direct cell printing. Then these units can be assembled to a larger tissue size by self-assembly or layering of cell sheets[92] to mimic the native microstructural repeating functional unit of the bone tissue. Bottom-up TE creates a more biomimetic engineered matrix at tissue level than the top-down approach.

6.3.2.2 Processing Techniques Micromolding and photolithography can be used to generate 3D cell-laden hydrogels. Micromolding of hydrogels provides a potentially powerful method for fabricating micro- and nanostructures.[93–95] Micromolding uses poly(dimethyl siloxane) (PDMS) molds microfabricated into a variety of shapes and sizes. In the first step, the prepolymer solution with the cell suspension is molded with PDMS mold. Then the solution is cross-linked by changing pH, temperature, ionic strength, or photoinitiator to generate a hydrogel with exact microstructures with the size and shape of the PDMS mold.[96] Many types of natural and synthetic hydrogels can be used for encapsulation of living cells, such as agarose,[97] chitosan,[98] and poly(ethylene glycol).[99] Collagen is a natural biocompatible and biodegradable material and has been extensively used to simulate the native ECM in tissues.

Photolithography provides another reliable technique to make microstructural modules with definite shapes, typically using photomasks with diverse patterns for patterning multiple cells in specific regions. Using this technology, a prepolymer solution of a cross-linkable hydrogel with photoinitiator is placed under a mask and is irradiated with ultraviolet light. The hydrogel cross-links only in the transparent areas of the mask to generate patterns similar to those of the mask. Khademhosseini and coworkers have intensively investigated the fabrication of cell-laden microgels for tissue engineering. They used this technique to create cell-laden microtissues and microfluidic devices.[100] Hydrogels can be patterned to create cellular microstructures for *in vitro* cell studies or 3D microtissues with biomimetic structures.

Because the complex architecture of most tissues is organized by assembly of repeating functional units over several scales, the cell-laden microgel units need to be assembled to larger structures at tissue level. Bottom-up assembly of cell-laden microgels has received increasing attention. These assembling techniques include random assembly,[101] manual manipulation,[102] multilayer photopatterning,[102,103] and microfluidic-directed assembly.[104,105]

Another approach is lamination of nanofiber layers with a hydrogel precursor solution followed molding to the final shape and cross-linking. In this approach, thin layers of nanofibers of PLGA, PLLA, PCL, or other polymers are produced by electrospinning. Then the fiber layers are laminated by compression molding using a hydrogel precursor solution containing bMSCs.[106] Then the laminated layer is wrapped around a cylindrical rod to form a microtubular osteon-mimetic structure and cross-linked by photopolymerization. The central canal in each microtube serves as a conduit for vascularization. A set of these microtubes can be adhesively bonded

to form a macroscale 3D cell-laden construct mimicking the microstructure of the cortical bone. This technique can potentially overcome the challenges associated with nonuniform cell seeding and vascularization and nutrient exchange within a bone-mimetic geometry.

However, these assembling techniques have their own drawbacks; they lack control over the final structure or lack scalability. Du et al. developed a more controllable assembling technique, which used hydrophobic effects in water–oil interfaces. Hydrophilic microgel building blocks microfabricated by photolithography were placed in hydrophobic medium and a secondary cross-linking reaction was performed.[105] However, this assembly technique exposed the microgels containing cells to the hydrophobic oil phase during the assembly procedure, which could influence cell viability.[105,107] Additionally, random or uncontrolled structures may form using this assembling approach. A recent work from Khademhosseini's group has modified the two-phase assembly technique using liquid–air interface of a hydrophobic solution to partially address the scaling-up issue by creating centimeter-scale cell-laden microgel assemblies.[92] However, this modified assembly was still performed in hydrophobic medium. To address this issue, directed assembly on hydrophilic templates was developed in the same group to fabricate 3D microgel constructs with a wide range of shapes and complexities such as tubes, spheres, and casques in 2D and 3D structures.[108,109] Other assembling techniques, such as physical templating and microfluidic-directed assembly, are also developed.[101,104,110]

Another novel technique in bottom-up TE approach is 3D cell, tissue, and organ printing. This technique is an attractive scaffold-free, rapid-prototyping based technology[111] with great potential for constructing delicate 3D tissue-like structures.[112,113] To engineer a bone tissue, osteogenic cell-laden hydrogels are deposited on a platform, yielding tissue constructs that consist of bone-forming cells and matrix at predefined locations within a porous 3D structure.[111]

Recently, Fedorovich et al. demonstrated the retention of spatially organized, functional osteogenic and endothelial progenitor cells, osteogenic matrix formation of hMSCs, and formation of erythrocyte-filled blood vessels in printed grafts after *in vivo* implantation.[114] SangJun Moon developed a bioprinter that used mechanical valves to print high-viscosity hydrogel precursor solutions containing cells within collagen, overcoming the problem of loss of cell viability and clogging in traditional inkjet printing systems.[115]

Cell bioprinting provides a potentially powerful technique in mimicking the native tissue microvasculature and microarchitecture, although the use of these implants still has limits in non–load-bearing applications. Temporary mechanical stability could be still required in combination with surgical instrumentation if applied in clinical environments.

Cell-sheeting techniques represent another bottom-up TE approach in which cells are grown on a thermo-responsive polymer substrate to secrete ECM and reach confluency. The confluent cell layer is detached by thermal regulation without enzymatic treatment, and single cell layers can be laminated into multiple single cell layers to form a thicker 3D matrix.[116] However, it is a challenge to construct thick tissues by this method because each layer is around 30 μm thick.[117,118]

Aside from the techniques discussed, cell aggregates are also a suitable building block for tissue-like constructs. The cell aggregates can be directly assembled into a tissue by using the adequate biological cues. Direct seeding of cell aggregates need to be in the presence of growth factors or other bioactive molecules to facilitate the dispersion of colonies into a larger cell construct.

6.3.2.3 Limitations and Challenges The bottom-up TE approaches hold great promise for creating functional repeating tissue units using hydrogels; they also provide a potential for assembling defined 3D microstructured modules for engineering tissue macroconstructs, which mimic the complexity of living tissues. However, random or uncontrolled structures still may form, so fabricating tissue constructs with biologically relevant length scales using the current setups is challenging.[119] Because of their high water content, hydrogels usually have poor mechanical stability. As a result, their use in constructing 3D tissues by bottom-up approaches is limited in load-bearing bone tissue. In addition, the control of the assembly process to fabricate 3D constructs with uniform shapes is still a challenge.

6.3.3 Novel Strategy (Integrating Approach)

6.3.3.1 Concept Top-down and bottom-up fabrication strategies have both advantages and disadvantages. The "challenges" of the bottom-up TE highlight the importance of scaffolds produced by traditional polymer processing techniques, such as porogen leaching and gas foaming. The lack of functionality of the top–down constructs underscores the importance of microenvironment for optimal cell growth. A combination of traditional top-down processes with more recent bottom-up microfabrication techniques may overcome this drawback and provide distinct advantages, bringing the field closer to the ultimate goal of complete control over microarchitecture and porosity in engineered tissues. The key question is how these two directions can be integrated. New strategies are still required to overcome the limitations of each of the current TE approaches.

6.3.3.2 Integrating Processes Mata et al. integrated top-down microfabrication with self-assembling peptide-amphiphile (PA) systems to offer a unique platform in which both physical and biomolecular elements were combined in a single material with cell behavior controlled by cell processing. In this integrated approach, bioactive scaffolds combine biologically instructive nanoscale fibers with topographical features to establish highly complex tissue structures.[120]

Ouyang et al. assembled a bMSC sheet on a knitted PLLA scaffold for engineering ligament analogs by a wrapping technique. Their results show that the approach of assembling bMSC sheets onto a knitted PLLA scaffold is promising for producing tissue-like and functional ligament analogs.[121]

Sargeant et al. developed a hybrid bone implant material consisting of porous Ti–6Al–4V foam and self-assembled PA nanofibers. Cells were encapsulated into the PA solution, and prewet Ti–6Al–4V foams with 52% porosity were placed in the PA and cell solution. PA solutions with cells were gelled with $CaCl_2$ to form nanofiber

matrices in Ti–6Al–4V foams. This hybrid bone graft, which integrated self-assembly of PA nanofibers within pores of metallic foams, has the potential to induce mineralization and direct a cellular response from the host tissue.[122]

Although the integration of bottom-up and top-down micro- and nanotechnologies brings new potentials to create tissue regeneration scaffolds with physical and biochemical hierarchical order from the micro- to macroscale, sophisticated technologies need to be developed. The major challenge of integration of bottom-up techniques with more traditional top-down approaches is to create more complex tissues than are currently achievable using either approach alone by optimizing the advantages of each technique.[123] Currently, there is no integrating technique that can be used to assemble complex hierarchical structures to meet the requirements of tissues and constructs, and research is now focused on targeting this problem.

6.4 FUTURE DIRECTION AND CONCLUDING REMARKS

Integration of a top-down TE approach with a bottom-up biological assembly concept is promising to engineer fully functional tissues and organs with micro- and nano biomimetic hierarchical complexity. Each approach has its own strengths and weaknesses and is suitable for different TE applications. The continuous development of top-down TE techniques will improve the scaffold's microstructure, presentation of cell signaling factors, and the interaction between multiple types of cells. The improvements in bottom-up approaches will generate novel self-assembling building blocks and complex larger scale tissue structures. With continued research in these advanced techniques, bone tissue engineering will advance toward clinical restoration of tissue function. Advances in top-down and bottom-up approaches will improve scaffold mechanical properties, cell–cell and cell–matrix interactions, and cell shape and morphology, leading to the formation of a vascular mineralized matrix in the damaged tissue and greater integration of the construct with the host vasculature.

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7

CHARACTERIZATION OF THE ADHESIVE INTERACTIONS BETWEEN CELLS AND BIOMATERIALS

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7.1 INTRODUCTION

Cellular adhesion is critical for many cellular functions, including spreading, proliferation, and migration. The interactions between cells and their environment are mediated by adhesion receptors located on the cell surface. Adhesive interactions can take place between cells or between a cell and the surrounding extracellular matrix (ECM). Adhesion receptors are responsible for both types of interactions. Biomaterials have been introduced as a means to facilitate cell adhesion and infiltration during the repair or replacement of damaged or diseased tissues. For biomaterials to successfully act as an alternative ECM, the interactions between cells and biomaterials must mimic the adhesive interactions in native tissue. Therefore, the control and optimization of adhesive interactions is an important aspect of material fabrication. Cell–material interactions can be regulated through material design and processing. This chapter focuses on the adhesion receptors responsible for the interactions that occur within native tissue, current biomaterial fabrication methods that attempt to mimic these interactions for tissue engineering applications, and measurement techniques that investigate cell–substrate and cell–cell adhesion strength.

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7.2 ADHESION RECEPTORS IN NATIVE TISSUE

Cells form connections with the ECM as well as with each other through adhesion receptors that are present on the surface of the cell. There are three major classes of adhesion receptors: integrins, cadherins, and members of the immunoglobulin (Ig) family. Although the exact functions of each class of receptor may vary, many adhesion receptors share some common properties, including the formation of receptor clusters after binding with an extracellular ligand and the formation of connections to the underlying cytoskeleton.

7.2.1 Integrins

Integrins are a superfamily of cell adhesion receptors that exist as 24 distinct transmembrane αβ heterodimers,[1] which can be found in Table 7.1. Currently, there are 18α and 8β subunits identified, which associate through noncovalent interactions.[2] The term *integrin* originates from the importance of such receptors at maintaining the "integrity" of the cytoskeleton. Integrins primarily interact with

TABLE 7.1 Integrin Heterodimers and Their Extracellular Matrix Binding Sites

β Subunit	α Subunit	ECM Binding Site
β_1	α_1	Laminin, collagen (GFOGER)
	α_2	Collagen (GFOGER), laminin, E-cadherin
	α_3	Laminin
	α_4	VCAM-1, ICAM-4, fibronectin
	α_5	Fibronectin (RGD)
	α_6	Laminin
	α_7	Laminin
	α_8	Fibronectin (RGD)
	α_9	VCAM-1
	α_{10}	Collagen (GFOGER), laminin
	α_{11}	Collagen (GFOGER)
	α_v	Fibronectin (RGD)
β_2	α_D	ICAM, VCAM-1, fibronectin, fibrinogen
	α_L	ICAM, ICAM-2, ICAM-4
	α_M	ICAM, ICAM-4, fibrinogen
	α_X	ICAM, ICAM-4, fibrinogen, collagen
β_3	α_v	Fibrinogen, fibronectin (RGD), ICAM-4
	α_{IIb}	Fibrinogen, fibronectin (RGD)
β_4	α_6	Laminin
β_5	α_v	Vitronectin (RGD)
β_6	α_v	Fibronectin (RGD)
β_7	α_4	VCAM-1, fibronectin
	α_E	E-cadherin
β_8	α_v	Vitronectin (RGD)

ECM ligands but also have the ability to interact with cell surface ligands. Integrins serve as a connection between the extracellular environment, where they bind to a ligand or adjacent cell surface, and the intracellular environment, where they bind to the cytoskeleton. Individual integrins may bind to multiple ligands, and multiple integrins can share the same ligand.[3] Integrin activation results in alterations of cell behavior (e.g., adhesion, proliferation, shape, survival or apoptosis, motility, gene expression, differentiation).[1] Because of the importance of integrin activation on cell function, biomaterials can be designed to mimic integrin interactions and achieve specific cell functions.

The function of integrins as transmembrane links between their extracellular connections and the cytoskeletal elements within the cell often plays an important role in mechanosensing. With the exception of $\alpha 6\beta 4$, which links to the intermediate filaments of the cytoskeleton, most integrins form intracellular connections with the actin cytoskeleton.[2] This anchoring function of integrins plays an important role in several cell functions, including blocking apoptosis and triggering the progression of the cell cycle.

Current research has supported the function of integrins in mechanotransduction, indicating that integrin activation and initiation of downstream signaling pathways can result in multiple cellular responses, including ECM remodeling, differentiation, and survival signaling. In cardiomyocytes, hemodynamic overload results in stimulation of cell growth and survival signaling.[4] Because of the stretch resulting from hemodynamic overload, integrin binding domains on the ECM become exposed, triggering integrin activation and the initiation of downstream signaling. Similarly, intracellular integrin activation can occur through the deformation of the underlying cytoskeleton because of stress.[5] Structural alterations of the actin-filamin cytoskeleton expose binding sites for the β tails of integrins, causing activation and stimulation of downstream signaling pathways.

Integrin activation as controlled through substrate stiffness has recently been shown to play a role in both osteogenic differentiation and tumor progression. The differentiation of mesenchymal stem cells into osteoblasts varied with the stiffness of the matrix, resulting in greater differentiation on stiffer substrates.[6] Additionally, a similar correlation was found for $\alpha 2$ integrin expression, indicating that this integrin subunit may play a role in transmitting mechanical signals into downstream signals for differentiation. This hypothesis was confirmed through a knockdown of $\alpha 2$ by siRNA that resulted in a downregulation of osteogenic differentiation.[6] The integrin $\alpha 5\beta 1$, which is important in the formation and remodeling of the fibronectin network of the ECM, has been shown to play a role in matrix stiffening and tumor progression. Increased matrix stiffening as a result of integrin activation was shown to accelerate tumor metastasis.[7] Understanding the mechanisms of tumor progression is critical for developing methods of prevention or treatment.

The role of integrins in mechanotransduction should be exploited in order to initiate or inhibit downstream signaling in response to integrin activation from mechanical stress. Biomaterial design, specifically material properties and three-dimensional structure, should address ways to promote integrin activation in situations when activation can lead to positive effects such as cell survival or

differentiation but also address means to inhibit integrin activation. Inhibition of integrin responses to mechanical changes that occur during the progression of cancer could have significant therapeutic implications. Nanoparticles that contain integrin-like particles within their membranes could bind to ligands on the ECM and prevent ECM ligand binding to integrins on the cell membrane, inhibiting integrin activation and the associated downstream effects. Additional studies on the signaling pathways involved in cancer progression may also reveal how to counteract integrin activation intracellularly. If there is an antagonistic signaling pathway that can be activated to stop downstream signaling or reverse ECM remodeling, therapeutic mechanisms could be designed to target the activation of those antagonist receptors.

Integrin molecules have been shown to cluster upon activation, particularly as a result of binding to a component of the ECM. Integrin clustering triggers the formation of focal adhesions, which are complexes that can transmit mechanical and regulatory signals. Focal adhesions are critical for several types of downstream signaling, including tyrosine phosphorylation, cellular pH elevation, enhanced phosphatidylinositol-4,5-biphosphate (PIP2) synthesis, and activation of the mitogen-activated protein kinase (MAPK) cascade, among others.[3] Focal adhesions provide a signaling platform that can mediate several subsequent reactions to ligand binding at once because of the proximity of activated integrins and their downstream effectors. Current research has implicated abnormalities in focal adhesion formation in several disease states, including some types of cancer, rheumatoid arthritis, and cardiovascular disease.[8] The assembly and disassembly of focal adhesions is not fully understood, but Rho GTPase is thought to play a crucial role.[3,9] The formation of focal adhesions is an important aspect of mechanosensing and it is generally thought that adhesive interactions that result from focal adhesions are stronger than those that are formed by a single ligand-receptor binding event. Engineering biomaterials to contain functional signaling platforms that promote focal adhesion formation could provide a means to study and control the signaling of cells *in vitro*.

In addition to being regulated by ligand binding, integrin function can be controlled intracellularly. For many integrins, the active state is not constitutive. These integrins exist on the cell surface in an "off" or inactive state in which no ligand binding or downstream signaling can occur until there is activation by an intracellular signal.[2] Platelet activation is an example of this type of integrin regulation. To be capable of binding to fibrinogen, von Willebrand factor (vWF), and fibronectin, the integrin $\alpha IIb\beta 3$ must be internally activated. Inside-out activation can occur through several different routes, including through thrombin, adenosine diphosphate (ADP), or epinephrine signaling, which function through G protein–coupled receptors; through signaling, which occurs through the vWF receptor; or through collagen signaling, which occurs through the collagen receptor and the integrin $\alpha 2\beta 1$.[2] Recently, it has been shown that the cytoskeletal protein α-actinin plays an important role in the "inside-out" signaling that activates the platelet ligand $\alpha IIb\beta 3$.[10] An understanding of integrin activation in platelets could lead to the development of drugs and biomaterials that can help initiate clotting from both the inside and the outside.

Some integrin–ligand pairings are more common than others, and specific sequences that appear frequently have been identified. Among the most common of these is the arginine-glycine-aspartic acid (RGD) sequence. Approximately one-third of integrins have binding sites for the RGD tripeptide, which can be found on many ECM proteins, including fibronectin, vitronectin, fibrinogen, and the latency-associated peptide (LAP) complex part of inactive transforming growth factor β. Although the RGD sequence is not readily exposed by collagen or laminin, there are cases in which denaturation or cleavage of these proteins results in exposure of the RGD sequence and subsequent integrin binding.[11] The RGD-binding integrins include all five of the αV integrins, two β1 integrins, and the αIIbβ3 integrin. RGD binding integrins can bind a large number of ECM and soluble vascular ligands. The ligands that contain the specific tripeptide active site bind with the integrins through an identical atomic basis.[12]

The affinity of integrins to the RGD sequence has been exploited extensively in tissue engineering research and therapy development. Recently, nanocarriers with RGD tethering on the surface have been shown to use the integrin–ligand specificity to target tumors that are rich with RGD-binding integrins.[13] Optimization of this drug delivery vehicle to increase the specificity, targeting, and loading efficiency of the nanocarrier can have a significant therapeutic impact. RGD peptides have also been shown to positively influence the differentiation of mesenchymal stem cells into articular chondrocytes[14] as well as the development of functional cardiac tissue from neonatal cardiac cells.[15]

Similar to the RGD sequence, the tripeptide leucine-aspartic acid-valine (LDV) is a common ligand among a group of integrins. LDV is an acidic motif that is functionally related to RGD and is suggested to bind to integrin receptors in a similar fashion.[16] LDV is present on fibronectin, and a related sequence is present on vascular cell adhesion molecule 1 (VCAM-1). The β2 integrins as well as α4β1, α9β1, and α4β7[9,11,16] contain a binding site for the LDV ligand.

Although RGD-binding integrins can recognize the RGD sequences that are exposed when collagen is degraded or cleaved, another specific amino acid sequence can be recognized by integrins when collagen structure is intact. The glycine-phenylalanine-hydroxyproline-glycine-glutamic acid-arginine (GFOGER) sequence exists on triple helical collagens. The sequence is recognized by a group of collagen-binding integrins, including α2β1, an important integrin in hemostasis. It is speculated that the GFOGER sequence is exposed once per microfibril unit of collagen.[11] The proximity of the ligand sequences on a microfibril of collagen promotes integrin clustering and focal adhesion formation, the importance of which has already been discussed.

Similar to collagen, binding sequences on laminin are only recognized by RGD-binding integrins if the ECM protein has been disrupted. The integrin binding sequence tyrosine-isoleucine-glycine-serine-arginine (YIGSR) has been discovered as the minimum sequence necessary to promote binding and adhesion between integrin receptors and epithelial cells on intact laminin.[17] YIGSR is found to be highly active in epithelial cells yet much less active in chondrocytes, osteoblasts, and fibroblasts.

7.2.2 Cadherins

Cadherins are a superfamily of glycoproteins that function to mediate cell-to-cell adhesions. Most cadherins are composed of an extracellular domain that sets up interactions among neighboring cells, a transmembrane domain, and a cytoplasmic domain, which is often linked to the elements of the cytoskeleton.[18] Cadherins are calcium-dependent molecules, and the calcium-binding domain is conserved throughout the various types of cadherins within the superfamily.[19] Because of the involvement of cell-to-cell adhesion in numerous cellular processes, cadherins have been found to contribute to cell signaling, recognition, and sorting in addition to cell adhesion. Alterations to normal cadherin function have been linked to several diseases, particularly cancer.

The cadherin superfamily can be divided into at least six subclasses. The oldest and most well understood are the classical cadherins: E-, N-, and P-cadherins as well as VE-cadherin. Classical cadherins are single spanning transmembrane proteins that primarily function in the formation of adherens junctions. Adherens junctions are typically located on epithelial cells and are formed by the interaction of classical cadherins.[20] E-cadherin is primarily associated with adherens junctions, but similar structures exist in a variety of epithelial cell types; for example, in squamous epithelial cells, both E- and P-cadherin independently form adherens junctions.[21] The cytoplasmic domain of adherens junctions bind to β-catenin or plakoglobin, which in turn bind to α-catenin. α-Catenin links the cadherin–catenin complex to the actin cytoskeleton either through direct binding to actin or indirect binding to vinculin, ZO-1, or α-actinin, which leads to actin binding.[20] Evidence has shown that lateral clustering of cadherins occurs in the formation of adherens junctions and that the redistribution of cadherin binding sites is a means to regulate cell adhesion as well as stimulate a stronger adhesion between cells.[22]

The classical cadherins also play a vital role in development. It has been shown that N-cadherin functions in neural development, including retina development and the formation of neural nodes and neural networks. Recently, attempts to mimic the N-cadherin structure present during neural development has shown promising results, including induction of the differentiation of neural stem cells and desirable cell–cell interaction.[23]

Desmosomal cadherins function in the formation of desmosomes and are one of the few types of cadherins that bind to the intermediate filaments of the cytoskeleton rather than actin. Desmocollin and desmoglein are the two subfamilies of the desmosomal cadherins. Tissues that undergo mechanical stress, such as the epidermis and the myocardium, are rich in desmosomes.[19] Structurally similar to adherens junctions, desmosomes link to the intermediate filaments of the cytoskeleton. Desmosomes are the result of a heterotropic interaction between one desmocollin and one desmoglein cadherin. The cytoplasmic domains of desmosomes directly link to plakoglobin, which binds to a second intermediate protein, desmoplakin.[24] Desmoplakin forms the connection between the cadherin complex and keratin intermediate filaments.[25] Similar to adherens junctions, evidence indicates that desmosomal cadherins cluster to form desmosomes.[26] Desmosome expression

has been found not only to be tissue specific but also cellularly specific within a single tissue, such as in different strata in the same stratified epithelial tissue.[27] Because of this specificity, it is hypothesized that desmosomes may play a role in epithelial tissue differentiation.

Other subclasses of the cadherin superfamily include the protocadherins, the 7TM-cadherins, the T-cadherins, and the FAT family of cadherins. Protocadherins are a very large family of cadherins that exhibit moderate adhesive activity. The major subfamilies of the protocadherins are μ-protocadherin and CNR-cadherin. Although the exact function of these cadherins is still unclear, it is hypothesized that they play a role in the development of the nervous system.[19] The 7TM-cadherins are a family of membrane proteins that contain seven transmembrane segments and function similarly to G protein–coupled receptors and have a large impact on cell adhesion related signaling. The Flamingo cadherin is one of the better studied 7TM-cadherins, and it is thought to have an important role in establishing the polarity of the cell.[19]

T-cadherins are the only type of cadherins that have no transmembrane or cytoplasmic domains. Rather, the T-cadherin is linked to the membrane through a glycosylphosphatidylinositol (GPI) anchor. GPI-anchored proteins are thought to be more densely located within lipid raft domains, which are known signaling platforms. Research in cardiomyocytes suggested that T-cadherins may also be located in lipid rafts and therefore function in cell signaling.[19,28] Cadherins in the FAT family have very large extracellular domains and are most highly expressed by proliferating cells that are undergoing development rather than in adult tissues. This observation has led to speculation that FAT cadherins have functions that are beyond cell–cell adhesion and are more closely related to cell migration and maturation during morphogenesis.[19]

Similar to the research that has been done to exploit integrin–ligand interactions for tissue engineering, the cell–cell interactions and downstream effects that result from cadherin activation could be of interest in many applications, including stem cell differentiation studies and engineering of epithelial and endothelial layers. Biomaterial design should consider the inclusion of cadherin-like particles on the surface to promote cell attachment and necessary interaction between cells.

7.2.3 Immunoglobulins

Immunoglobulins are a superfamily of membrane proteins that share a common domain referred to as the Ig fold motif.[29] Igs have been found to have an important role in the activation and regulation of the immune system because immune cells must be nonadherent when circulating the blood and lymph but become adherent when migrating through tissue.[30]

There are three main subfamilies of Igs, which function similarly in different tissue types. The intracellular cell adhesion molecule (ICAM) family are type I transmembrane glycoproteins that contain two to nine Ig domains. ICAM-1 is expressed constitutively in venular endothelial cells and some leukocytes and can be stimulated by cytokines.[29] ICAM-1 can serve as a ligand to some integrins, which

is an important part of immune system activation. ICAM-1 has recently been used as a way to attract stem cells to an area of injury on the endothelium. By coating the surface of mesenchymal stem cells with antibodies to ICAM-1, Ko et al.[31] were able to successfully target the interaction between the stem cells and the endothelium. ICAM-2 is expressed constitutively on platelets and endothelial cells but unlike ICAM-1 is not affected by cytokines. ICAM-4 is expressed on erythrocytes.

VCAM is a transmembrane protein that contains six or seven extracellular Ig domains. Similar to ICAM-1, VCAM can also function as a ligand for integrins and is responsive to cytokines.[29] VCAM can be expressed on both vascular and nonvascular cells and is an important mediator in some cell signaling pathways. Junctional adhesion molecules (JAMs) are also type I transmembrane proteins that contain two extracellular Ig domains and are found in the tight junctions of endothelial and epithelial cells.[32] JAM proteins are known to form homodimers and have an important role in the trafficking of leukocytes.

Research in biomaterial development for tissue engineering applications aims to mimic the native adhesive interactions that are mediated by integrins, cadherins, and immunoglobulins. Controlling the interactions between cells and the underlying substrate offers a means to control the downstream effects of cell adhesion, which includes cell spreading, proliferation, and migration. In the following section, modifications that intend to optimize these interactions are discussed.

7.3 OPTIMIZATION OF CELLULAR ADHESION THROUGH BIOMATERIAL MODIFICATION

Degradable polymeric scaffolds are typically used *in vitro* and *in vivo* in the field of tissue engineering and serve as a temporary matrix that can be seeded with cells to promote healing, proliferation, and differentiation at an injury site. Polymeric scaffolds must meet certain criteria before being used: they must have degradation, mechanical, adhesive, and biocompatible properties that will result in proper healing and regeneration of tissue at the implant site.[33] Some of the commonly used natural and synthetic polymers are discussed briefly in this chapter, but for more thorough information on the properties these materials, please see Tables 7.2 and 7.3 for a list of materials as well as suggested references for further information.

Several factors play a role in how cells adhere and respond to biomaterials. On a basic level, the hydrophilicity of a material has an effect on cell adhesion. In a study completed by Schakenraad et al.,[34] several commonly used polymers were tested, and the results showed that those with a higher degree of hydrophilicity better supported cell adhesion than those that were hydrophobic. Although cells may prefer a hydrophilic polymer *in vitro*, biomaterials always exist *in vivo* in the presence of a protein solution. In the native environment, cells rarely interact with biomaterials directly but instead interact with an adsorbed protein layer on the surface of a material. Protein adsorption is also related to the hydrophilicity of a material. Highly hydrophilic materials resist adsorption and therefore resist cell adhesion *in vivo*.

TABLE 7.2 Commonly Investigated Natural Biomaterials for Tissue Engineering Applications

Material	Key Reviews	Current Research
Polysaccharides		
Agarose	33	76–78
Alginate	51	52–54
Hyaluronic acid	79	80–82
Chitosan	83	84–86
Polypeptides		
Collagen	87	88–91
Gelatin	92	93–95
Silk	96	97–99

Poly(ethylene glycol) (PEG) is an example of a hydrophilic synthetic polymer that resists protein adsorption. This quality is exploited for applications in which cell adhesion is not ideal. As a linear chain, PEG has poor overall material properties, but as a network, its properties are greatly improved. PEG is nondegradable *in vivo*, but this shortcoming can be overcome by copolymerization. Copolymerization with degradable moieties such as lactic acid has been shown to result in degradation of these modified PEG scaffolds. In such scaffolds, the bioactive moieties degrade rapidly, breaking apart the PEG polymer into monomer degradation products.[35] Although the degradation of modified PEG varies based on the material chemistry, average rates tend to reach 100% degradation after the first month after implantation.[35] Modifications and copolymerization of PEG is common for tissue engineering applications. For example, a modified PEG hydrogel has recently been used as an injectable scaffold for cartilage tissue engineering.[36] Copolymerized scaffolds

TABLE 7.3 Commonly Investigated Synthetic Biomaterials for Tissue Engineering Applications

Material	Key Reviews	Current Research
Polyesters		
Poly(glycolic) acid	100	101–103
Poly(L-lactic) acid	104	105,106
Poly(D,L-lactic acid-*co*-glycolic acid)	107	47–50
Poly(ε-caprolactone)	108	109–111
Poly(propylene fumarate)	112	113–115
Polyorthoester	116	
Other		
Polyanhydrides	117	118,119
Polyphosphazenes	120	121–123
Polycarbonates	100	124,125
Poly(ethylene glycol)/poly(ethylene oxide)	126	36,37,127,128
Polyurethane	129	130,131

composed of an alginate–PEG combination have shown promise in islet of Langerhans encapsulation, with the double cross-linking properties of the scaffold allowing for better scaffold stability.[37]

The adsorption of ECM proteins onto the surface of biomaterials is one of the simplest means to improve cell attachment to a biomaterial and can be increased by using the use of culture medium that contains serum. In general, the major components of serum are albumin, vitronectin, and fibronectin. Protein absorption on a biomaterial surface as a mediator of cell adhesion has been demonstrated extensively in the literature.[38–41] A study conducted using a hydrophobic self-assembled monolayer was incubated in a fibronectin solution before cell seeding and showed that because of to the fibronectin adsorption, fibroblasts were able to strongly adhere to the scaffold and maintain adhesion under applied shear stress conditions.[42] In addition to serum, a more specific protein solution can be used to pretreat a tissue engineering scaffold to promote cell adhesion. Use of a specific protein solution adds an additional layer of control or targeting and can result in interactions between the cell and the material, which can in turn mediate desired downstream effects on cell behavior.

One method of optimizing cell adhesion to a tissue engineering scaffold is to incorporate adhesion motifs within the scaffold composition. As mentioned earlier, known adhesive domains such as RGD and YIGSR are present within the ECM and promote adhesion in the native environment via integrin binding. Interactions between the cell and one of these domains can promote anchorage, migration, and signal pathway activation, which in turn mediates numerous intracellular reactions.

To exploit these adhesive domains for tissue engineering applications, short bioadhesive peptides have been tethered onto the surface of synthetic and natural polymers. Molecules are typically tethered through the use of PEG or poly(ethylene oxide) polymer spacers so that the bioactive molecule can be presented to a cell.[43,44]

Bioadhesive peptides can be derived from natural or synthetic sources, each with advantages and disadvantages. Naturally derived bioadhesive peptides have been successfully used in biomimetic material studies but are very difficult to isolate and purify, especially while maintaining functionality. Because of this shortcoming, synthetic bioadhesive peptides are commonly fabricated and used for biomimetic applications.[45]

The inclusion of bioadhesive peptides has shown significant enhancement of cellular activities.[44,46] Peptides are typically tethered in a random yet spatially uniform manner across the surface of the biomaterial. Recently, studies have shown that if the peptides are arranged within clusters, the cellular response is increased.[46] As discussed earlier, integrin clustering occurs during adhesive interactions to promote stronger or multiple simultaneous downstream effects. In a study investigating how cell adhesion with an orthopedic implant can be used to promote better tissue integration, Petrie et al[46] showed that clustering of bioadhesive ligands on the surface of the implant upregulated osteogenic signaling and differentiation of human mesenchymal stem cells.

Polyesters are a commonly used type of synthetic material (see Table 7.3) that typically undergoes hydrolytic degradation. Poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), and poly(D,L-lactic acid-*co*-glycolic acid) (PLGA) are polyesters and

are among the most widely used synthetic polymers.[33] PLGA is a copolymer of PLA and PGA. The copolymer is amorphous and exhibits a faster degradation rate and lesser mechanical strength than PLLA alone depending on processing. PLGA undergoes bulk degradation as a result of ester hydrolysis, the rate of which can be controlled by altering the ratio of PLA and PGA in the copolymer. PLGA has been used extensively and has been shown to support new tissue growth in several bone tissue engineering applications.[47–49] PLGA has also been shown to promote restoration of function when seeded with neural stem cells *in vivo*.[37,50]

Polyesters are typically nonadhesive materials, but their material properties offer ideal conditions for many tissue engineering materials such as smooth muscle cell culture. To overcome the adhesion limitation, Ilagan and Amsden[44] tethered RGD sequences to the surface of the polyester material through the use of a PEG spacer. The results of the study showed that inclusion of the bioadhesive peptides significantly improved cell adhesion and proliferation.

In addition to the use of a polymer spacer, nanopatterning is another means to tether bioadhesive peptides to the biomaterial surface. Fabrication is achieved through a number of techniques, including self-assembly, self-assembling monolayers, stamping, and nanoprinting.[45] Alginate is a natural polymer that is commonly used for nanopatterning studies. Alginate forms a hydrogel when exposed to divalent ions, such as calcium. Gelling is easily reversed by sequestering ions through the use of a chelating agent.[51] Alginate offers limited cell attachment without modification, although this has been shown to be ideal for the culture of hepatocytes, resulting in native cluster formation and albumin production *in vitro*.[52] Alginate has also been used in studies with fibroblasts, in which the cells were found to maintain their function for a prolonged period of time within an alginate sponge.[53] Similarly, chondrocytes have been found to maintain their native phenotype more effectively in the three-dimensional environment provided by alginate.[54]

Because of the inert nature of alginate, it is a popular candidate for modification by nanopatterning the surface with tethered bioadhesive peptides. In a study conducted by Comisar et al.,[55] an alginate hydrogel was coupled with RGD, which was nanopatterned into "high-density islands." Results showed that the pattern of the islands elicited different cellular responses. Whereas focal adhesion kinase (FAK) phosphorylation, an important marker for focal adhesion formation and cell spreading, was most responsive to closely patterned islands, osteogenic differentiation occurred when islands were farther spread apart.

Similar studies have investigated how the density and placement of adhesive peptides as well as other bioactive molecules such as proteins and growth factors affects the behavior of cells that are seeded onto modified scaffolds.[43,56,57] Because adhesion receptors cluster upon ligand binding, closely packed patterns of bioactive molecules tend to elicit different cellular responses than those that are more spread out. Nanopattern fabrication can also be achieved through the use of a self-assembled monolayer (SAM). Self-assembled monolayers are typically formed using thiol molecules assembled in a designated pattern onto a substrate such as gold or glass.[58] Bioactive molecules have been successfully attached to the thiol molecules and have been shown to mediate changes in cell behavior such as proliferation and differentiation.[43,57]

In addition to integrin binding domains, cadherins have also been attached to the surfaces of biomaterial scaffolds and have been shown to promote angiogenesis.[45] There are still some inherent limitations with the use of adhesive oligopeptides, however. Synthetic peptides have much lower activity than that of the native ligands and have limited specificity. There are also several conformational differences between the native adhesive domains and the synthetic ones, which can similarly result in lower adhesion activity and specificity.

The physical topography of a biomaterial surface can also influence the adhesion of cells to the surface. The topography of a material has the ability to create strict sites of cell adhesion. Similar to tethering bioadhesive peptides, the creation of sites for cell–material interactions more closely mimics the *in vivo* environment of the tissue. Contact guidance is the ability of cells to spread with directionality, which is often dictated by the topography of the material.[59] Micro- and nanofabrication techniques have been used to exploit native contact guidance.

An example of micro topography used in tissue engineering applications is the use of patterned co-cultures in the creation of tissue-engineered constructs. Patterned co-cultures allow for control over the degree of contact, including cell–cell contact as well as cell–material interactions, and are created through a variety of possible microfabrication techniques, including photolithography, microfluidics, and polydimethylsiloxane (PDMS) stencils.[60] Parallel grooves have also been used to promote a variety of adhesive interactions. Receptor-binding domains can be concentrated on the raised portions of the grooves so the adhesive receptors on cells can better recognize the domains and mediate the formation of focal adhesions. ECM elements can be directed to the parallel grooves of the scaffold, promoting a highly organized cell–ECM environment *in vitro*. In a similar fashion to the parallel groove topography, the capillary network has been mimicked by using fabrication techniques that alter the biomaterial topography. The capillary network was reconstructed using a highly porous elastomer scaffold that contained a parallel array of channels. Neonatal rat heart cells were cultured within these channels and showed better contractile properties after the 8-day study.[61]

The consideration of cell–substrate interactions in biomaterial design has resulted in the successful creation of materials that are able to elicit downstream cellular responses such as differentiation and proliferation. In the following section, methods to quantify cell–substrate interactions are discussed. Quantification of cell adhesion can provide another means to characterize the interactions between cells and an underlying substrate.

7.4 MEASUREMENT OF CELL ADHESION

The adhesion of cells to an underlying substrate can be quantified through the use of cell adhesion assays. In general, cells are allowed to establish adhesive interactions to a substrate of interest and then are exposed to a detachment force. Adhesion assays are often categorized based on the type of force that is applied, resulting in three major categories: micromanipulation, centrifugation, and hydrodynamic shear

stress.[62,63] Currently, the vast majority of cell adhesion measurements are studied in two-dimensional systems. In the future, the field would benefit greatly from the development of quantitative assays that could characterize cell adhesion in a three-dimensional environment because this is a more relevant configuration and representation of the native tissue.

Before the development of assays based on detachment force, "stick and wash" assays were commonly used for the study of cell adhesion.[63,64] In a stick and wash assay, cells were allowed to adhere to a surface and then were simply subjected to washing over the surface with buffer. Although many of the first discoveries involving cell adhesion ligand–receptor interactions were made using this technique, there are inherent limitations. Stick and wash assays had poor reproducibility and applied uneven and unknown detachment forces.[63,64] These limitations led to the development of the measurement techniques discussed next.

7.4.1 Micromanipulation

In micromanipulation techniques, the detachment force can be applied as either a vertical force pulling cells normal to the surface or a shear force, pulling cells tangential to the surface.[62] Micromanipulation covers a range of techniques that include micropipette aspiration, atomic force microscopy (AFM), and laser tweezers.[63] With these techniques, it is possible to collect real-time force-displacement measurements on a single cell and investigate specific interactions between cell adhesion receptors and the substrate.

AFM can be used to evaluate morphologic changes occurring during cell adhesion, adhesion strength measurements, and interaction forces between cells. AFM images are capable of showing cell flattening and spreading, and it is generally accepted that the flatness of a cell designates good adhesion (Fig. 7.1).[65] A typical morphologic change related to adhesion is the appearance of structured stress fibers, indicating the stability of the cells on the underlying substrate. The adhesion strength of cells to an underlying substrate can be quantitatively measured on a single cell level using AFM techniques. The force necessary to laterally displace a cell with the

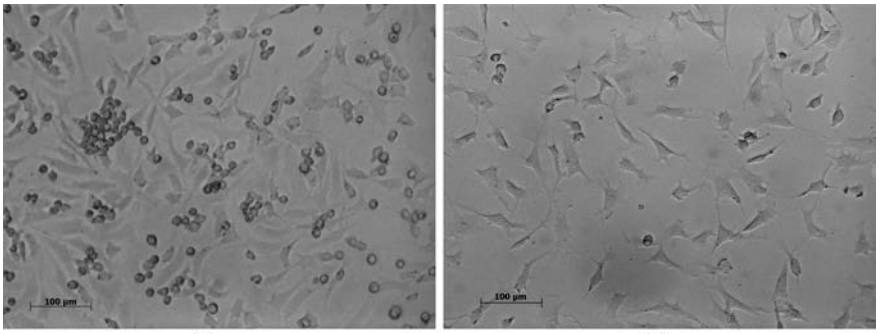

FIGURE 7.1 Morphologic assessment of adhesion.

AFM cantilever can be measured in real time by recording the deflection of a laser beam versus the lateral displacement of the AFM cantilever.[65] In addition to measuring the adhesion strength, the AFM technique can also estimate the timescale over which adhesion occurs and eventually saturates.

As previously mentioned, the interaction between cells is mediated by cell adhesion molecules called cadherins. The interaction forces between cells can also be evaluated at the molecular level using AFM. Single biomolecules can be imaged, and the force necessary to disrupt cell–cell interactions can be characterized.[65]

AFM techniques can have several practical difficulties.[66] The underlying substrate as well as surrounding cells can influence the force measurements that are collected from a single cell. Additionally, the user must be sure that the forces being measured are the attachment forces of the cell and not simply a measure of cell membrane strength. Other practical issues include z-axis restrictions and protein adsorption to the AFM cantilever.

Micropipette aspiration is another type of micromanipulation technique that is capable of measuring the strength with which a single cell or even a single biomolecule on a cell surface is attached to an underlying substrate. The displacement force used by micropipette aspiration is suction pressure, which can be applied tangential or normal to the cell surface[62,67,68] and can be designed in various ways. The micropipette can be designed to simply apply suction pressure onto a cell that is attached to a substrate, resulting in partial or complete removal of the cell from the surface and into the pipette tip. It can be designed so that the cell detaches from the substrate and attaches instead to a bead that is held by suction force at the tip of the probing pipette, or it can be designed such that a cell is freely moving inside one pipette until it attaches to a bead or cell held by a second pipette.[69]

In a study by Athanassiou and Deligianni,[62] vertical (normal) suction forces were applied to individual bone marrow cells that had been allowed to attach to fibronectin. To establish a suction force normal to the cell surface, the tip of the micropipette was bent at a 130-degree angle. The results showed that detachment occurred in phases. First, deformation was observed, without detachment of the cell, followed by a second phase in which detachment was observed as a result of pressure increases. The strength of the adhesion of bone marrow cells to fibronectin was found to increase as the time allowed for cell attachment was increased.

Qin et al.[68] also used micropipette adhesion as a means to quantify the interaction of tenocytes grown *in vitro* to fibronectin and type I collagen modified PLGA. In this study, the suction force of the micropipette was applied tangential to the surface of the cell, although the results of the study were quite similar. With tenocytes, the adhesion strength increased as seeding time increased. In both studies, soluble antibodies were used to disrupt cell adhesion by inhibiting ligand–receptor binding. Both studies showed that inclusion of competitive soluble antibodies decreased cell adhesion strength to the respective substrates.[62,68]

Micromanipulation assays offer a sensitive and quantitative means to investigate cell–substrate and cell–cell interactions at the molecular level. However, these assays are limited to applying small forces and can only be used for individual cell studies in which the seeding time is short. For longer adhesion times or for quantification of a

larger cell population, assays that provide a greater distractive force must be considered.

7.4.2 Centrifugation

For larger cell populations, centrifugation assays offer a simple and reproducible means to quantify cell adhesion. In general, cells are seeded onto a substrate and allowed to adhere for a period of time, typically no longer than 1 h.[63] After adherence, cells are subjected to a perpendicular detachment force generated by spinning at a specified speed in a standard laboratory centrifuge.[63] A schematic of a typical centrifugation assay procedure can be seen in Figure 7.2. The ratio of post-spin cell count to pre-spin cell count results in the adherent fraction of cells at the designated force set by the centrifugal speed.

Centrifugation assays have also been used to quantify differences between initial adhesion and "strengthened" adhesion.[64] Strengthened adhesion is defined as adhesion that occurs while cells are incubated on a substrate. As with the micromanipulation assays, adhesion strength increased with longer seeding times (e.g., 30 vs. 60 min),[64,70] showing that adhesion is time dependent. Reyes and Garcia[70] further modified the centrifugation assay and developed the mean adhesion strength value, which is the force that causes 50% cell detachment, for fibrosarcoma cells seeded on fibronectin coated 96-well plates. Centrifugation assays offer a simple and reproducible method of characterizing biomaterials based on the ability of the material to successfully initiate cell adhesion.

Despite the success of centrifugation assays, there are limitations, including the fact that only one speed can be subjected to cells at a time and that at longer adhesion times (>1 h),[63] the distraction forces generated by the centrifuge are not large enough to displace large cell populations.

7.4.3 Hydrodynamic Shear Stress

Flow systems have been developed to apply a wide range of shear detachment forces to large adherent cell populations and are generally considered a more reliable adhesion measurement system.[63] Hydrodynamic shear stress assays are classified according to the geometry of the flow responsible for generating the shear detachment

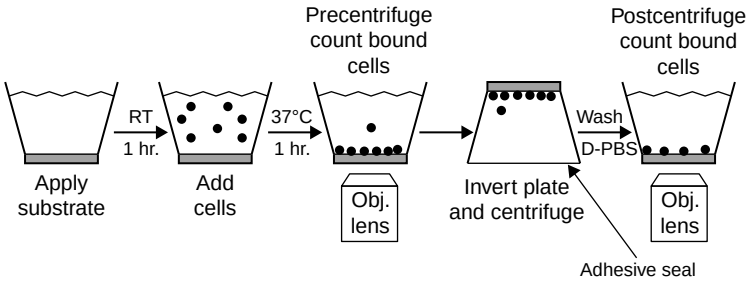

FIGURE 7.2 Schematic of a centrifugation assay procedure.

force. There are three basic flow cell configurations: the parallel plate, the rotating disc, and radial flow between parallel disks. Of these geometries, the parallel plate has been extensively studied, specifically in combination with microscopy.[63] The parallel plate configuration permits the observation of attachment and detachment throughout the assay and has been used frequently in the characterization of leukocyte–endothelial cell adhesion events.[71,72] A typical parallel plate flow cell is designed so that (1) flow is laminar (Reynolds number <2300) and controlled through the use of a syringe pump and (2) the entrance length is minimized so that entry effects can be neglected, and flow can be considered fully developed and parabolic.[72]

The rotating disk geometry results in forces that vary linearly with radial distance, which is an advantage because it can subject a large cell population to a range of detachment forces in one experiment. Common types of rotating disks are the single spinning disk and the small-gap parallel disk viscometer.[63] The rotating disk should only be used for low rotational speeds because increasing the speed results in a greater degree of unsteady and invalidated flow rates. Garcia et al.[73] investigated the use of the single spinning disk configuration on fibronectin-mediated osteosarcoma adhesion to bioactive glass. For a given rotational speed and laminar flow, velocity, temperature, and concentration, boundary layer thickness can be considered constant.[74] The spinning disk was shown to produce reproducible results that demonstrated that cell detachment increased with shear force.[73]

Similar to rotating disk configurations, radial flow systems can also generate a range of shear stresses. In this case, surface shear stress decreases with radial distance. Through the use of immunoglobulins, this geometry has been used to characterize ligand–receptor interactions over a range of forces.[75]

Although hydrodynamic shear stress assays provide a reliable and reproducible means for quantifying cell adhesion *in vitro*, there are some limitations related to the measurement of shear stress. In general, the adhesion strength is reported as a shear stress with units of force per area. Even though this is a useful measure for the investigation of adhesion strength, the net force that is applied is not simply the shear stress but includes parameters such as hydrodynamic drag and torque.[63] Therefore, results of such assays must be carefully examined. There are several practical difficulties of hydrodynamic shear stress assays, including complications with the system setup, preventing the inclusion of air bubbles in the stream of flow, and preventing nonlaminar flow through the chamber.[66]

All of these adhesion assay techniques have advantages and disadvantages, making it clear that there is no perfect solution when it comes to quantifying cell adhesion. A measurement system must be chosen based on the cell system in place and the desired results. Results of adhesion assays must be taken as relative to the cell population and the particular experiment and not as an absolute measure.

7.5 CONCLUSIONS

Adhesion receptors function to modulate cell behavior in a variety of ways, and these functions are desirable to incorporate into the design of biomaterials that will be used for tissue engineering applications. A common method is the tethering of integrin

binding domains such as RGD, LDV, GFOGER, and YIGSR onto the surface of the biomaterial to promote cell adhesion. The inclusion of adhesion receptor ligands into biomaterials enhances cell adhesion and can potentially mediate a desired intracellular reaction to ligand binding.

Research involving the modification of biomaterials to optimize cellular adhesion has made strides including bioactive molecules on the material surface to control cell phenotype and induce changes such as increased spreading, proliferation, and differentiation. Patterning techniques have allowed for control over adhesive domain inclusion on the surface of biomaterials at the micro- and nanoscale. Optimizing biomaterial design to mimic the native adhesive interactions of a cell population provides a method for controlling downstream cell responses and could have significant impacts for therapeutic applications.

The quantification of cell adhesion through the use of a detachment force offers a means to further characterize and optimize cell–cell and cell–substrate interactions. Results of such assays can be used as feedback for biomaterial designs and encourage further manipulation of biomaterials to achieve desired levels of cell adhesion.

By controlling the adhesive interactions through biomaterial design, future studies can focus on the exploration and characterization of the intracellular mechanisms by which the cell response occurs after activation of adhesion receptors. Better understanding of the exact signaling mechanisms could provide invaluable information on cellular development and the progression of disease within a cell. This information could be used in the development of replacement tissues and drug delivery devices to treat diseases such as cancer. Finally, to make the leap from the benchtop to the clinic, the scalability and stability of bioactive scaffolds should be addressed in order to produce an efficient means for applying such devices for tissue replacement and therapeutic interventions.

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DISCLAIMER

The items identified by brand name are the choice of the authors and are not to be construed as conveying an official endorsement by the U.S. Food and Drug Administration.

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8

MICROFLUIDIC FORMATION OF CELL-LADEN HYDROGEL MODULES FOR TISSUE ENGINEERING

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8.1 INTRODUCTION

Engineering of functional living tissues *in vitro* is important in regenerative medicine for generating transplantable grafts and pharmacokinetic studies. To engineer functional living tissues, cells need to be in the same environment as *in vivo*; *in vitro* tissues should mimic both the physical and the chemical properties of *in vivo* tissues.[1] *In vivo* tissues have three-dimensional (3D) hierarchical structures composed of various types of cells and extracellular matrices (ECMs). In these structures, cells receive mechanical stress and chemical stimuli from other cells and the surrounding ECMs.[2] These properties should be incorporated into engineered tissues to reconstruct an *in vivo*-like environment.

Hydrogels are commonly used for cell cultures because they are mostly a biocompatible, biodegradable, hydrophilic material and have a porous matrix.[3] These characteristics of hydrogels allow culturing of cells under relatively mild conditions. Moreover, because the size, shape, porosity, and chemical properties of hydrogels can easily be designed according to specific requirements, hydrogels are

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ideal materials for providing *in vivo*-like environments.[4] Therefore, hydrogels are useful for creating 3D cell structures.

Recently, microfabricated hydrogel modules are used as attractive microenvironments when culturing living cells; these hydrogel modules allow cells to attach on their surfaces or to be encapsulated (or both).[3,5] Such cell-laden hydrogel modules are mostly prepared by using microfludic devices because these devices can manipulate liquids in a controlled manner with high throughput, high uniformity, and design flexibility.[5–7] Because the size uniformity of the cell-laden hydrogel modules facilitates their handling, the modules are used in a wide range of applications, including the parallel analyses of cellular functions in the microfluidic analytical systems and the reconstruction of controlled-dense 3D cellular structures in "bottom-up" tissue engineering.[8,9]

This section provides an overview of (1) fabrication methods and characteristics of cell-laden hydrogel micromodules, (2) handling techniques of the modules in microfluidic devices, and (3) applications of the modules for transplantation and bottom-up tissue engineering.

8.2 CELL-LADEN HYDROGEL MODULES

This chapter introduces the reproducible fabrication methods of cell-laden hydrogel modules with a controllable design and the characteristics of the modules.

8.2.1 Types of Hydrogels

The favorable gelling mechanisms in microfluidic devices are ionic cross-linking with multivalent counterions, covalent cross-linking, and inherent phase transition such as heat of transition.[10] For example, hydrogels gelated under mild conditions are best suited to produce hydrogel modules for encapsulating cells. By selecting hydrogels with the appropriate gelling mechanism, hydrogel modules with the desired functions are obtained.

Hydrogels are mainly categorized into two types: hydrogels based on natural polymers and hydrogels based on synthetic polymers. Among the hydrogels obtained from natural polymers, collagen, hyaluronate, fibrin, alginate, and agarose are potentially attractive for tissue engineering applications. Collagen and hyaluronate are tissue-derived natural polymers and components of the ECMs. Collagen gelates in response to temperature change. Hyaluronate gelates by covalent cross-linking with hydrazide derivatives and radical polymerization of glycidyl methacrylate. Fibrin is a protein found in blood and gelates by the enzymatic polymerization of fibrinogen. Alginate and agarose are plant-derived natural polymers obtained from algae. Alginate gelates with divalent cations such as Ca^{2+} and Ba^{2+}. Agarose forms thermally reversible gels.

Among hydrogels obtained from synthetic polymers, poly(ethylene glycol) (PEG) and polypeptides have attractive potential applications. PEG gelates using by various methods, and a type of PEG is a photocross-linkable hydrogel. Moreover,

polypeptides are synthetic proteins that can mimic natural proteins. Genetically engineered polypeptides can be synthesized while controlling for their properties such as functions, stiffness, degradation, and cellular interactions. By considering the gelation, mechanical, and chemical properties of hydrogels, we can select suitable hydrogels for desirable applications.

8.2.2 Microfluidic Devices for Hydrogel Module Production

Many groups have demonstrated the production of hydrogel modules with high throughput and uniformity, and advances in microfluidic devices have resulted in various configurations of hydrogel modules such as blocks, beads, tubes, and sheets. The microfabrication techniques used for producing the hydrogel modules are reviewed in this section.

8.2.2.1 Hydrogel Beads A conventional method to produce hydrogel beads is emulsification. In this method, an immiscible liquid mixture of sol solution and oil with surfactants is stirred to generate small sol droplets.[11] The major disadvantages of emulsification are large-size distribution and more damage to encapsulated cells than the other approaches discussed here.

By using microfluidic devices, monodisperse and small sol droplets are produced. To generate sol droplets, quasi-two-dimensional (2D) planar microfluidic devices, such as T-junction microchannels[12,13] and 2D microfluidic flow-focusing devices,[14] are often used. In T-junction microchannels, monodisperse sol droplets can be formed at the T-junction facing two immiscible liquids such as a sol solution and oil including surfactants because the oil–surfactant mixture breaks the sol solution into droplets (Fig. 8.1a–c). In 2D flow-focusing devices, an inner fluid is broken into droplets by an outer fluid at the orifice or the downstream channel (Fig. 8.1d). When the sol solution is used as the inner fluid and oil with surfactants is used as the outer fluid, 2D flow-focusing devices produce monodisperse sol droplets. The droplet formation in both devices is driven by the Plateau–Rayleigh instability.[14,15] Therefore, droplet size is easily controlled on the basis of flow rates and channel dimensions. Additionally, both devices have the ability to produce sol droplets with high uniformity (coefficient of variation, CV) (<5%) and high throughput. However, the type of droplets that can be produced is dictated by the surface chemistry of the channel walls (i.e., hydrophobicity or hydrophilicity) because the inner fluid comes into direct contact with the channel walls; this phenomenon is known as the wetting problem. Therefore, sol droplets cannot be stably formed in hydrophilic channels because of the wetting problem. In some cases, fouling of channel surfaces by proteins and carbohydrate might make the formation of even sol droplets in hydrophobic channels difficult.[13] To circumvent the wetting problem, it is necessary to change the surface chemistry of 2D microfluidic channel walls (e.g., silanization) to cater for the desired usage.[16]

To avoid the wetting problem without any chemical modification, various 3D axisymmetric flow-focusing devices (AFFDs) have been developed to form monodisperse droplets.[17–20] In an AFFD, an inner fluid is surrounded by an outer fluid, and

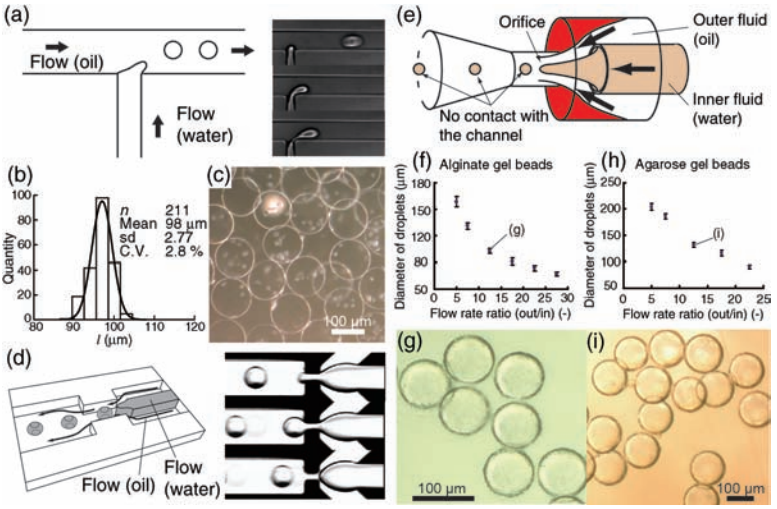

FIGURE 8.1 (a) Schematic illustration of the formation monodisperse droplets using the T-junction microchannel and images of droplet formation at the T-junction taken by a high-speed video camera. (b) Size distribution of the alginate gel beads produced by the T-junction device. The alginate gel beads show monodispersity (CV = 2.8%).[13] (c) Monodisperse alginate gel beads encapsulating Jurkat cells. The beads are also of a narrow size distribution.[13] (d) Schematic view of the 2D flow-focusing device and images of droplet formation in a flow-focusing device. In the 2D flow-focusing device, the droplets produced come into contact with the top and bottom of the channel walls. Therefore, the wetting problem occurs. (e) Schematic diagram of an AFFD used to produce monodisperse droplets. AFFDs can prevent droplets from adhering to the surface of the channels because the droplets are always surrounded by the outer fluid.[18] (f) Plot of the sizes of the alginate gel beads in the oil versus the flow rate ratio (outer flow rate/inner flow rate). CVs of every point are less than 5%, indicating all beads produced by the AFFD are monodisperse.[18] (g) Image of alginate gel beads in oil corresponding to (g) in figure (f).[18] (h–i) Formation of monodisperse agarose gel beads. The graph and images are in the same sequence as the alginate gel beads.[18] (e–i) Copyright (2009) Springer.

droplets are formed at the orifice or the downstream channel (Fig. 8.1e). The inner fluid and the produced droplets are always surrounded by an outer fluid, allowing the monodisperse droplets not to contact the channel walls, thus eliminating the wetting problem. The AFFD can control the size of droplets by adjusting flow rates and the orifice dimensions because droplet formation in an AFFD is controlled by the Plateau–Rayleigh instability (Fig. 8.1f–i).[17] Moreover, similar to 2D microfluidic devices, an AFFD can produce sol droplets with high uniformity and high throughput. After gelation of these monodisperse sol droplets produced by the microfluidic devices, monodisperse hydrogel microbeads are obtained.

Alternatively, monodisperse hydrogel microbeads are obtained using microjetting.[21] Using an inkjet printing technology, monodisperse sol droplets are produced in air, and the droplets are gelated to hydrogel microbeads by submerging them in an initiator of gelation. Also, the inkjet printing technology can organize hydrogel

microbeads into a layered formation in a solidifying gel with sequential hydrogel microbead fusion. The microjetting method can form not only hydrogel microbeads but also structures of other shapes, such as sheets and tubes, by stacking the hydrogel microbeads.

8.2.2.2 Hydrogel Blocks To produce hydrogel blocks, photolithography, flow lithography, and micromolding techniques are mainly used. Photolithography has a fast and high-resolution ($\sim$1 μm) performance in forming hydrogels with various shapes and sizes.[22] In the photolithography technique, the sol solution is initially molded into block shape and polymerized by using ultraviolet (UV) light. Because UV light triggers gelation, photocross-linkable microgels such as PEG should be used in this technique. The plane shape of the hydrogel blocks is obtained from the photomask design, and their depth follows the depth of the mold. Moreover, the plane shape of the depth direction can be controlled by changing the focus depth of UV light and the photomask patterns (Fig. 8.2a).[23] Therefore, photolithography can produce complicated structures such as polygons and rotundate blocks.

Alternatively, photolithography combined with microfluidics, so-called "flow lithography", allows the production of multifunctional hydrogel modules in continuous flow with high throughput.[24,25] Hydrogel blocks are fabricated in a microchannel by direct polymerization of UV light through a photomask. Then hydrogel blocks are obtained in continuous flow. The shapes of the hydrogel blocks are determined by the photomask patterns (Fig. 8.2b). In addition, computer-aided flow lithography is an advanced method to flow lithography.[26] In this technique, a computer controls the lighting areas to generate exposure patterns, and floating hydrogel blocks are obtained at arbitrary times and positions.

Micromolding is a simple method for producing hydrogel blocks with controlled features.[27] By using photolithography, silicon, glass, and polymer molds can be obtained in various sizes and shapes. Soft lithography has especially enabled the easy fabrication of poly(dimethylsiloxane) (PDMS) molds. To form hydrogel blocks of a variety of shapes and sizes, the sol solution is inserted into micromolds and subsequently gelated using an arbitrary gelling mechanism. The hydrogel blocks have high-resolution performance because the blocks transcribe the micromold patterns.

8.2.2.3 Hydrogel Fibers and Tubes Hydrogel fibers and tubes can be generated by cylindrical flow of solutions inside microfluidic devices. Transferring a cylindrical flow stream of a sol solution into an initiator of gelation can easily produce hydrogel fibers (Fig. 8.2c–e).[28] Also, hydrogel fibers are fabricated by coaxial microfluidic flows comprising a core flow surrounded by a sheath flow. When a sol solution and an initiator of gelation are introduced into the core flow and the sheath flow, respectively, a hydrogel fiber is formed.[29] Meanwhile, when a sample flow is concentrically located between the core flow and the sheath flow, hydrogel tubes are formed when the sol solution is inserted into the sample flow and the initiator of gelation is run into the core flow and the sheath flow (Fig. 8.2f and g).[30] The hydrogel fibers and tubes are formed without length limitation, and their diameter can be controlled by the flow

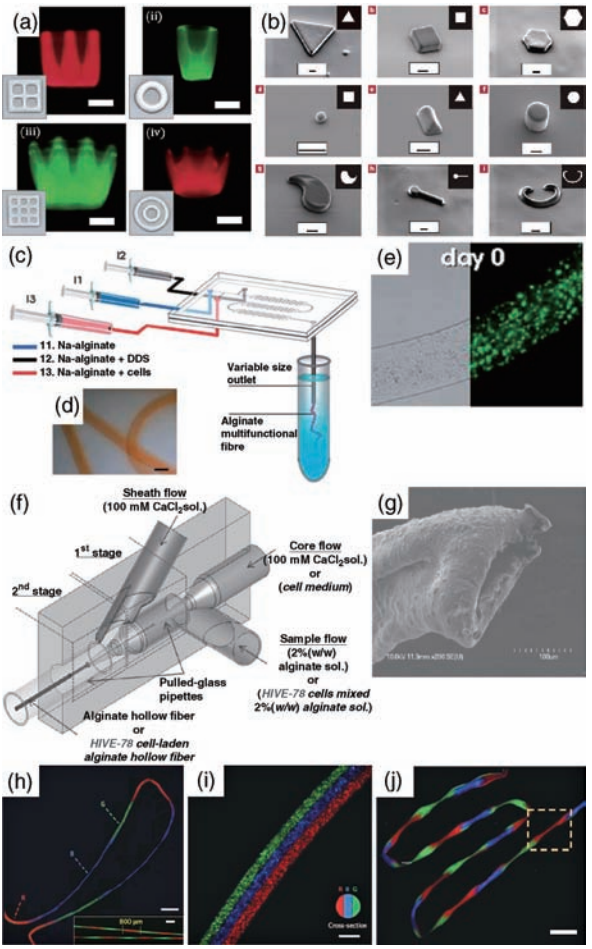

FIGURE 8.2 (a) Image of 3D-pattern hydrogel blocks fabricated by photolithography. The plane shape and depth of blocks are not only changed by the photomask design and the mold depth, respectively, but the plane shape of depth direction is also controlled by the altered focus depth of the UV light and light path. Scale bar, 50 μm.[23] (b) Image of hydrogel blocks formed by flow lithography. The shape of blocks can be easily varied according to the photomask design. The scale bar for all panels is 10 μm.[25] (c) Schematic fabrication procedure of hydrogel fibers.[28] (d) Image of the alginate fiber. Scale bar, 200 μm.[28] (e) Optical bright field and fluorescence image of alginate fiber containing Wharton's jelly mesenchymal stem cells.[28] (f) Schematic diagram of the microfluidic device that generates the alginate gel tubes. Coaxial flow composed of core and sample flows is generated, and gelling of the alginate sol in the sample flow starts at the interface of both flows. After the sheath flow surrounds the coaxial flow, solidification occurs simultaneously at the interface between the sample flow and the sheath flow.[30] (g) Scanning electron microscopic image of the hydrogel tube.[30] (h) Image of serially coded fiber and its close-up image. Scale bars, 1 mm and 400 μm (close-up image).[31] (i) Image of parallel coded fiber. Scale bar, 200 μm.[31] (j) Image of a fiber with spatiotemporal variations in morphology and chemical composition. Scale bar, 1 mm.[31] (h–j) Each type of fluorescent polystyrene beads emits different colors: red, blue, and green. (a) Copyright 2011 Royal Society of Chemistry, (b) copyright 2006 Nature Publishing Group, (c–e) copyright 2011 Royal Society of Chemistry, (f, g) copyright 2009 John Wiley and Sons, and (h–j) copyright 2011 Nature Publishing Group.

rates and channel dimensions. In addition, by combining the fabrication techniques of hydrogel fibers and air valves, hydrogel fibers coded with varying chemical compositions and topographies along the fibers are generated (Fig. 8.2h–j).[31] Using this method, hydrogel fibers encapsulating multiple and spatially controlled cells can be created for tissue engineering applications.

8.3 CELL ASSAY SYSTEMS USING MICROFLUIDIC DEVICES

Because of their size uniformity, the cell-laden hydrogel modules are manipulated as monodisperse beads. Therefore, these modules can be handled, arrayed, and retrieved using microfluidic bead-based assay systems for accurate analyses. The characteristics of microfluidic devices for handling hydrogel modules and their potential for use in analysis of cell biological systems are reviewed in this section.

8.3.1 Microfluidic Devices for Handling Modules

Cell-based microfluidic devices can provide precise spatial and temporal control of samples and reagents, which is difficult to achieve using 2D cell culture systems. Moreover, they allow real-time monitoring and analysis of samples in order to obtain insight into cell dynamics. Microarray systems especially have extensive applications for drug discovery and diagnostic and basic scientific studies.

8.3.1.1 Microwell Devices Microwell devices have a simple mechanism to trap samples statically in an array.[32,33] They allow samples to sink into microwells and hold a sample in each microwell. Microwell devices are fabricated accurately using a variety of technologies such as photolithography. Microwells have the advantage of having a massively parallel format (>10000) and allowing the observation of all samples from the same direction. In addition, microwells are suitable for trapping a single sample in almost all microwells by adjusting the well diameters and depths. Therefore, microwell devices are used to screen samples for drug kinetics studies.

8.3.1.2 Hydrodynamic Microarray Devices Hydrodynamic microarray devices are commonly used for trapping samples in microfluidic systems. The following are the advantages of hydrodynamic microarrays over microwell devices: hydrodynamic microarrays allow transporting of samples, immobilizing of samples for convenient analysis, delivering reagents to samples with continuous observation, and retrieving of selected samples.

The most common way to immobilize samples in microfluidic systems is to arrange the side channels in a main channel.[34] When the diameter of the side channel is sufficiently small, samples are trapped by suction because the sidestream from the main channel runs samples into the side channel. The hydrodynamic systems are capable of releasing samples as the sidestream is reversed. Also, using hydrodynamic trapping holes, similar to the microwells, samples can be immobilized and retrieved

in large quantities. Thus, hydrodynamic microfluidic devices have an attractive potential for trapping and analyzing samples.

Single sample-trapping systems with individual addressing are developed using advanced microfluidics strategies;[35,36] meander-shaped hydrodynamic microfluidic devices are a representative example.[37,38] A meander-shaped hydrodynamic microfluidic device is composed of a meander-shaped main channel and a trapping region with a narrow, straight channel (Fig. 8.3a and b). The straight channel has lower flow resistance than the main channel when samples are not trapped. This phenomenon causes the bulk of the fluid to flow along the straight channel, and samples in the main stream are delivered into all trapping regions. Also, the meander-shaped hydrodynamic microfluidic device can retrieve a trapped sample (Fig. 8.3c). A laser setup to heat a localized area results in selective microbubble formation near the trapping area, and the expanding microbubble pushes the immobilized sample into the main stream. In the device, samples are selectively released, and the displaced samples can be collected by allowing them to flow out of the device. Moreover, a resettable configuration added to the meander-shaped hydrodynamic microfluidic device allows the release of all the trapped samples without clogging the device when the flow direction is reversed.[39]

8.3.2 Cell Analysis Using Microfluidic Devices

Using microfluidic devices, samples can be transported, immobilized, and observed continuously. For example, owing to the meander-shaped hydrodynamic microfluidic devices, monodisperse hollow alginate capsules coated with poly-L-lysine (PLL) containing motile cells can be immobilized and arrayed (Fig. 8.3d).[40] The hollow and semipermeable capsules facilitate the continuous observation of encapsulated arrayed motile cells while preventing invasion of other cells and allowing the exchange of nutrients and wastes.

Also, microfluidic devices allow investigating cells in 3D culture conditions. Cellular signals are sometimes changed in cells cultured on a flat substrate because cells adhered on a 2D substrate lack structural cues. Therefore, cell analyses in 3D culture systems are important for accurately understanding cell biology. Cell-laden hydrogel modules are a useful 3D culture model system because of their stable size, shape, and cell density. In addition, ECMs can be used as cell-encapsulating materials to construct tissue-like microstructures. Monodisperse alginate gel beads encapsulating Jurkat cells fabricated by the T-junction microchannel, and monodisperse collagen gel beads seeded with fibroblast cells (3T3 cells) formed by AFFD have been delivered and arrayed using a meander-shaped microfluidic channel.[38,41] Specifically, live-dead assay of fibroblast cells on collagen gel beads can be performed in a microfluidic channel during the beads trapping. In addition, a coculture assay system for constructing cell–cell interaction among different types of cells has also been realized in an improved meander-shaped microfluidic channel (Fig. 8.3e and f).[42] Because the channel can trap and pair different types of beads, the system would be applicable for continuous observation of cell–cell interactions between paired cell-laden hydrogel beads.

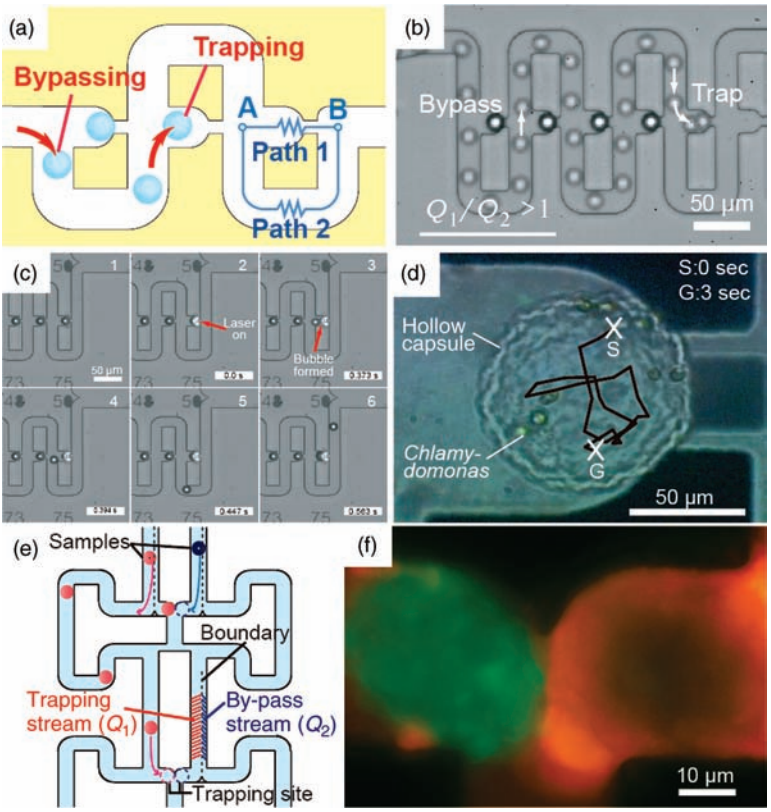

FIGURE 8.3 (a) Schematic diagram of a meander-shaped hydrodynamic microfluidic device. When the trapping area is empty, a bead in the mainstream is delivered into the trapping area. When the trapping area is full, beads are carried along the meander-shaped main channel.[37] (b) Superimposed time-lapsed high-speed camera image showing the trapped beads.[37] (c) Sequence images taken by a high-speed camera indicating the retrieval of beads using bubbles.[37] (d) Image of monodisperse hollow capsules comprising a PLL-alginate membrane and the encapsulating microbe, *Chlamydomonas*. The path traced by *Chlamydomonas* in 3 s is shown. The image shows that the motility of the encapsulated microbes is not compromised inside the hollow capsule. In addition, continuous observation was carried out for more than 2 h.[40] (e) Schematic view of the improved meander-shaped hydrodynamic device that allows different types of hydrogel beads to be paired.[42] (f) Fluorescent images of collagen gel beads with stained cells paired with different types of cells. *Red* indicates HepG2 cells and *green* indicates 3T3 cells.[42] (d) Copyright 2009 Royal Society of Chemistry and (e, f) copyright 2010 Royal Society of Chemistry.

8.4 IMPLANTABLE APPLICATIONS

Successful cell transplantation without immunosuppression might be achieved by immunoisolation through hydrogel encapsulation. The characteristics of cell-laden

hydrogel modules for transplantation and implantable applications are reviewed in this section.

8.4.1 Cell-Laden Hydrogel Modules for Transplantation

The approach for the immunoisolation of encapsulated cells uses a semipermeable membrane made from hydrogels. The membrane forms a mechanical barrier separating encapsulated cells from the host antibodies (>150 kDa) and immune cells but allows the diffusion of small molecules (<10 kDa) such as glucose, insulin, nutrients, and cell waste products.[43] Cells encapsulated into hydrogel beads are commonly used for implantation. Their spherical shape enables sufficient diffusion of nutrients and cell products because the bead shape have a better surface–volume ratio than materials of any other shape. Additionally, hydrogel beads cannot be easily disrupted, are mechanically stable, are reproducible by microfluidics methods, and can be implanted into the patient by a simple injection procedure.

Hydrogels for use in transplantation need to be immunoisolated and capable of diffusing nutrients and cell products. Alginate, agarose, and chitosan as natural polymers, and poly(hydroxyethyl methacrylate-methyl methacrylate) (HEMA-MMA), acrylonitrile, and PEG as synthetic polymers have reported uses for cell encapsulation in hydrogels.[44,45] Alginate-based beads are mainly preferred because alginate does not interfere with cellular functions and can keep arbitrary shapes.[46]

The fabrication process of alginate beads can be broadly classified into two categories: external gelation and internal gelation.[15] In the external gelation method, Na-alginate droplets are gelated by the external addition of divalent cations. For example, in the production of Ca-alginate beads, Na-alginate droplets are transferred into a calcium chloride ($CaCl_2$) solution. The rapid gelling behavior of alginate in the external gelation method makes it difficult to produce well-defined, homogeneous, and monodisperse alginate hydrogel beads. On the other hand, the internal gelation method involves dispersing an insoluble (or slowly soluble) complex in the Na-alginate solution.[47] Because of pH reduction, divalent cations are released from the complex, cross-linking the alginate to form homogeneous and monodisperse hydrogel beads.

The type of divalent cations cross-linking the alginate also contributes to the morphology of the alginate gel and the viability of the encapsulated cells. Ca ions are suited for the production of perfectly spherical and stable alginate beads. However, Ca ions have toxic effects, and encapsulated cells are damaged when they are exposed to Ca ions for a prolonged time. Ba ions react with alginate in a stronger way compared with Ca ions. Although Ba ions have high biocompatibility and cells encapsulated in Ba-alginate beads show high viability, Ba-alginate beads are easily deformable.[48]

8.4.2 Implantable Applications of Cell-Laden Hydrogel Modules

Hydrogel beads are used in cell transplantation (Fig. 8.4a): various types of cells (e.g., islet and Sertoli cells) encapsulated in alginate beads are transplanted into

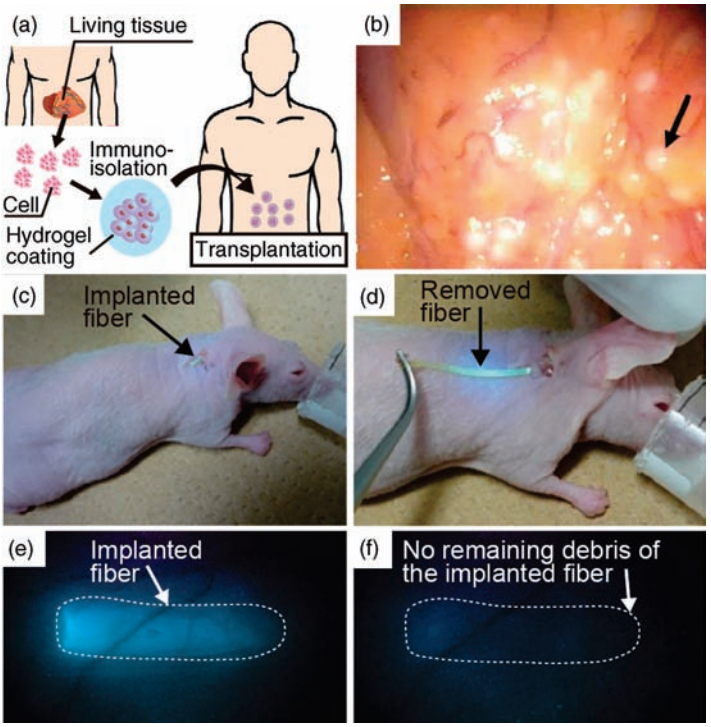

FIGURE 8.4 (a) Schematic diagram of transplantation using cell-laden hydrogel modules. Because hydrogel protect the invasion of immune antibodies, encapsulated cells can live for a long time without immune reactions. (b) Laparoscopic view of the omentum of a patient with type 1 diabetes 9 years after transplantation of alginate capsules encapsulating porcine islets.[49] (c) Fiber is implanted in the mouse ear to continuously monitor the glucose level.[50] (d) The implanted fiber is easily removed from the ear.[50] (e) Fluorescent image of the mouse ear containing the fiber. The fiber transmits fluorescent signals continuously depending on blood glucose concentration.[50] (f) Fluorescent image of the mouse ear after fiber removal. The image shows that the implanted fibers are retrieved from the ear without leaving debris.[50] (b) Copyright 2007 John Wiley and Sons.

living organisms. Encapsulation of cells protects them from immune antibodies and mechanical stress, so the cells remain viable for a long time (>1 year) after the transplantation.[48] Moreover, because the encapsulated cells express the cellular functions, alginate-based beads containing cells have potential to be considered as new therapeutic devices. For instance, islet cells encapsulated in alginate beads can control blood glucose levels *in vivo* for a long period and thus show potential to provide treatment for patients with diabetes (Fig. 8.4b).[49]

Recently, cells encapsulated in hydrogel tubes and fibers have been proposed.[28,30,31] The fibers and tubes have advantages over beads for long-term implantation *in vivo* (i.e., they can remain at the implantation space for a long period, but beads disperse from the implantation area). Also, fibers and tubes can be

easily and nonsurgically removed from the body by withdrawing their end portions. In addition, use of hydrogel fibers encapsulating a fluorescent gel indicates changes of glucose concentration (Fig. 8.4c–f).[50] By implanting the fibers, the glucose level can continuously be monitored *in vivo*. This shows the potential of hydrogel fibers and tubes for implantation, encapsulating not only cells but also various functional products.

Cell sheets are formed by incubating cells on a hydrogel layer that is used to detach the cells from a substrate. In particular, using a poly(*N*-isopropylacrylamide) (PNIPAAm) layer is a potentially powerful method for generating cell sheets.[51] Because PNIPAAm has a low critical solution temperature of 32°C and cells detach from the PNIPAAm layer under this temperature, cells cultured on a PNIPAAm sheet can be harvested as cell sheets along with their deposited ECM. Because the cell sheets keep the ECM on their basal surface, they can be transplanted directly to host tissues. Cell sheets have potential applications for cell-based regenerative therapies.[52] Transplantation of a single cell sheet into a host tissue can reconstruct corneal epithelium, skin, bladder urothelium, and periodontal ligaments. By homotypic layering of cell sheets, 3D tissue structures such as cardiac and smooth muscles can be recreated. With heterotypic stratification of various cell sheets, more complex and higher ordered laminar structures such as liver lobules and kidney glomeruli can be constructed. The use of cell sheets allows attaching cells to host wound tissues without loss of some cells.

8.5 TISSUE ENGINEERING

A demand currently exists for constructing macroscopic 3D tissue architectures that mimic tissue structures *in vivo* for repairing injured, degenerated, and inherently defective tissues. There are two different approaches for tissue engineering: the top-down approach and the bottom-up approach. The top-down approach uses 3D scaffolds with the desired tissue architecture.[53] In contrast, the bottom-up approach uses microtissue units as building blocks.[9] Microtissue units are powerful tools for reconstructing organomimetic and homogeneous dense structures. By molding microtissue units, uniform and arbitrarily shaped tissues can be fabricated without necrosis.

8.5.1 Microtissue Units

Microtissue units based on hydrogel modules are applicable for microscopic tissue engineering. One approach uses hydrogel modules encapsulating cells and covered by another type of cells. This method achieves formation of a hierarchical 3D coculture system for fabrication of microscopic tissues and heterotypic cellular interactions under 3D coculture conditions (Fig. 8.5a and b).[41,54] Because cellular functions are increased in the 3D coculture microtissues, microtissue-based hydrogel modules are useful for high-throughput studies of pathological and physiological phenomena in 3D coculture cells.

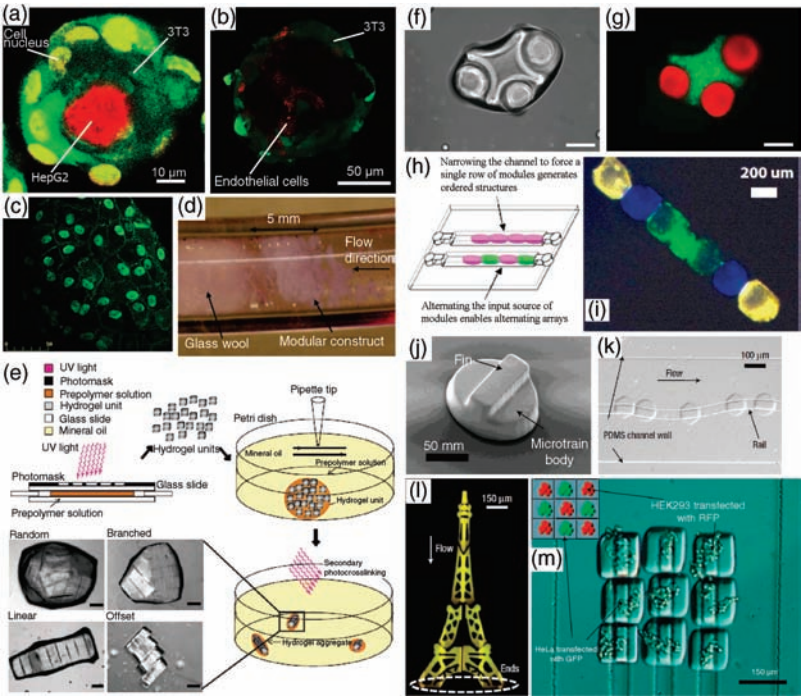

FIGURE 8.5 (a, b) Fluorescence confocal microscopy of hierarchical cocultured cell beads. (a) Collagen gel beads encapsulating HepG2 cells (red) and seeded with 3T3 cells (green) after 17 h of incubation.[41] (b) Self-assembling peptide beads containing endothelial cells (red) and 3T3 cells (green) after 2 days of culture.[54] (c) Fluorescent image of a collagen gel rod seeded with HUVEC cells showing the rod covered with a confluent cell layer.[55] (d) Image of the modular construct, including the microporous body.[55] (e) Schematic suggesting assembly of cell-laden hydrogel blocks in oil. Mechanical agitation applied using a pipette tip and secondary cross-linking form diverse shapes of gel assemblies: random, branched, linear, and offset.[56] (f, g) Images of the lock-and-key-shaped gel assembly containing three rods per cross. Cross-shaped gel and rod-shaped gel encapsulates red-labeled and green-labeled 3T3 cells, respectively. Scale bars, 200 μm.[56] (h) Schematic diagrams of the assembly of cell-laden hydrogel blocks to form lined-shaped cell structures in a microfluidic device.[57] (i) Image of the line-shaped cell structure consisting of three differently labeled cells.[57] (j) Scanning electron microscope image of the microtrain.[58] (k) Guided movement of microtrain along a reentrant rail.[58] (l) Image of the Eiffel Tower assembly produced by the guided assembly method.[58] (m) Image of the rail-guided assembly including two different types of living cells. *Green* and *red* indicate HeLa and HEK293 cells, respectively.[58] (c, d) Copyright 2006 National Academy of Science, (e–g) copyright 2008 National Academy of Science, (h, i) copyright 2008 Royal Society of Chemistry, (j–m) copyright 2008 Nature Publishing Group.

8.5.2 Random Assembly of Microtissue Units

Random assemblies of microtissue units result in vascularized tissues (Fig. 8.5c and d).[55] For this application, collagen gel rods seeded with endothelial cells (HUVEC cells) and encapsulating hepatocyte cells (HepG2 cells) are used. After preparing rod-shaped collagen gels encapsulating hepatocyte cells, endothelial cells are spread on the gel and cultured until a confluent layer of endothelial cells grows on the surface of the gel. The rod-shaped HUVEC-HepG2 microtissues are assembled into a larger tube with perfusion of medium or whole blood. The perfusion induces remodeling of cells and produces a perfusable tissue with a microporous body because the spaces between microtissues become microchannels. The simple method using a random assembly creates functional tissue equivalents and potentially engineer organ grafts.

8.5.3 Controlled Assembly of Microtissue Units

In contrast to random assembly, a controlled assembly method can make arbitrarily shaped tissues. In this method, the surface tension force at the water–oil interface is used to aggregate hydrogel blocks (Fig. 8.5e–g).[56] First, PEG-methacrylate (PEGmA) gel blocks containing cells are produced by photolithography using UV light. After gelling, the PEGmA gel blocks soaked in sol solution are transferred into mineral oil, where the gel blocks are assembled by mechanical agitation. Subsequently, exposure to UV light gelates the gel blocks into assembly. This method also generates 3D cell structures containing multiple types of cells. In particular, lock-and-key assemblies composed of cross-shaped gel blocks and plural rod-shaped gel blocks form coculture tissues without any additional steps. Using this method, the tissues formed from microtissue units are fabricated reproducibly without complicated assembly and handling procedures.

In the approach using microfluidic devices, collagen blocks containing cells fabricated by a micromolding method are assembled into microchannels or microchambers (Fig. 8.5h–i).[57] When assembling collagen blocks in microchambers, 3D cell structures are generated, but hydrodynamics in the narrow microchannel forces the collagen blocks to produce ordered structures. The advantage of this method lies in its ability to form tissue structures containing multiple types of cells. The 3D cell-lined structure comprising hierarchical coculture tissues will allow studying 3D cell–cell interactions and signaling.

In another approach, convex PEG gel blocks, microtrains, are produced by flow lithography and are fluidically assembled along a concave rail (Fig. 8.5i and k).[58] Using the rails as guides, complex 2D structures are built by fluidically assembling microtrains with zero error and incorporating all microtrains as components in the structures (Fig. 8.5l). Furthermore, heterogeneous fluidic assembly of microtrains containing different types of living cells is achieved by the same guiding mechanism (Fig. 8.5m). The guided and fluidic assembly method require the convex hydrogel blocks and the concave rails but has strong potential, owing to its flexibility, to produce heterogeneous and complex cell-laden structures as living tissues.

8.5.4 Macroscopic Assembly of Microtissue Units

Although various approaches using cell-laden hydrogel modules provide 3D cell structures, formation of a large-scale macroscopic tissue structure has been difficult because of the size, uniformity, and throughput limitations of hydrogel modules. One method of engineering macroscopic tissue structures is proposed as a solution: a random assembly approach consisting of monodisperse cell-laden collagen gel beads to achieve a rapid construction of millimeter-thick complex macroscopic tissues (Fig. 8.6a–c).[41] The tissue fabrication process uses monodisperse collagen gel beads fabricated by an AFFD and covers them with cells (namely, "cell beads"). The cell

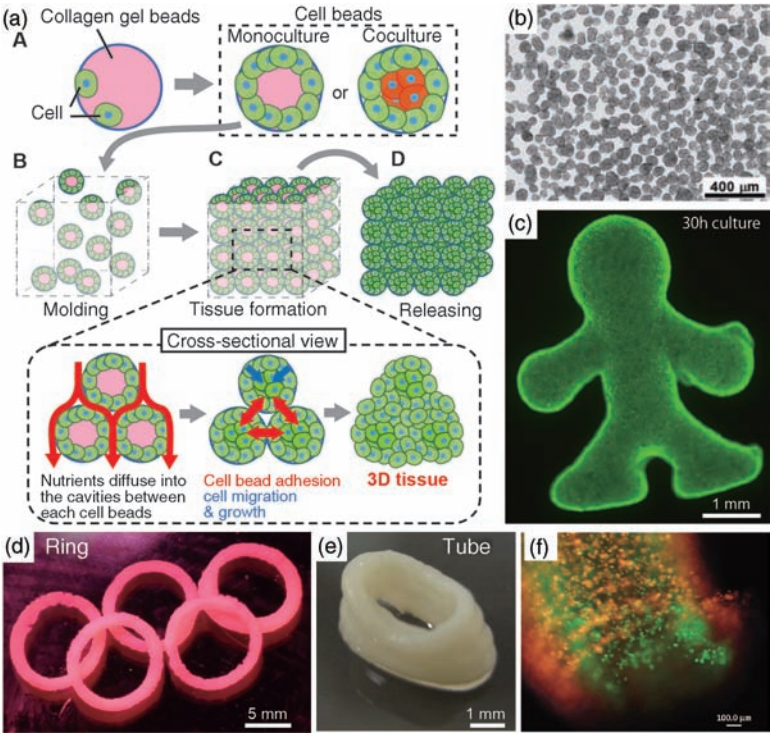

FIGURE 8.6 (a) Concept of bead-based tissue engineering: monodisperse cell beads are molded into a macroscopic cell structure.[41] (A, B) Monodisperse cell beads are poured into a designed PDMS mold. (C) During tissue formation, the medium diffuses inside the 3D cell structures via the cavities of the cell beads, supplying nutrients to all cells. (D) Macroscopic 3D cell structures are released from the PDMS mold. (b) Image of cell beads seeded with 3T3 cells; they are the building modules for the construction of a macroscopic 3D cell culture.[41] (c) Fluorescent image of the macroscopic structures having a complicated shape. Live-dead assay staining indicates that almost all cells within the structure are alive.[41] (d, e) Ring- and tube-shaped cell structures fabricated by the printing method.[41] (f) Tube-shaped hierarchical coculture structures made from printing cells. The inner layer is composed of human umbilical endothelial cells (green), and the outer layer is composed of human aortic smooth muscle cells (red). Image provided by Prof. Nakamura, Toyama University, Japan.

beads are then molded into a designed PDMS chamber to construct the macroscopic 3D tissue structure. In the chamber, the cells grow and migrate into the body of the beads by degrading collagen, and the cell beads adhere to each other via the cells enclosing the collagen gel beads, resulting in macroscopic 3D tissue structures. Finally, the fabricated tissue is removed from the PDMS mold. This method features the rapid production of macroscopic 3D tissue structures, a homogeneous cell density, and tissue formation without necrosis during less than 1 week because the cell culture medium can be supplied via the cavities between each cell bead and from the collagen. These characteristics make the method compatible with other 3D tissue engineering tasks because cell beads containing various types of cells can be easily assembled and aligned to generate macroscopic 3D structures with any desired shape.

The printing method is useful for a macroscopic controlled assembly approach for engineering 3D structures.[59] Printing generates microtissue unit composed of cells within hydrogels. In contrast to microtissue units fabricated by photolithography and flow lithography, printing can stack cells within hydrogels in layered form to construct 3D tissue structures. The advantage of the printing approach is the fast construction of 3D tissue structures with control of cell placement and structure geometries. Also, the printing method can create complex-shaped structures such as hollow-tube tissues (Fig. 8.6d and e).[41,60] Moreover, because the printing method can accurately control the location of cells in patterns, hierarchical tube structures containing two different types of cells are obtained (Fig. 8.6f).[61] Using the printing method, 3D structures containing various types of cells are built into the tissue matrix in a simple and versatile way.

8.6 SUMMARY

Microfluidic techniques succeed in reproducibly constructing diversely shaped hydrogel modules such as beads, blocks, fibers, tubes, and sheets with high throughput, high uniformity, and design flexibility. These hydrogel modules have applications in various fields ranging from basic biology studies to tissue engineering. Combined with cell assay microfluidic systems and cell-laden hydrogel modules, microtissues with ECM are useful for analyses of cell functions and cell–cell interactions because microtissues can easily be handled, arrayed, and retrieved in microfluidic systems. Furthermore, the cell-laden hydrogel modules can be used as building units for reconstructing 3D cell structures. Therefore, the cell-laden hydrogel modules produced by microfluidic devices have a great potential to create miniaturized tissues for human implantation and for treatment of diseases.

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9

MICRO- AND NANOSPHERES FOR TISSUE ENGINEERING

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9.1 INTRODUCTION

The emergence of regenerative medicine has resulted in a novel interdisciplinary field that focuses on repair, replacement, and regeneration of diseased or damaged tissues or organs.[1–3] Despite extensive efforts in the past several decades, only limited success has been reported for synthetic biomaterials in the clinical setting, and autologous and allogeneic tissues are still widely accepted as the "gold standard" for tissue regeneration therapies. As one of the most important strategies in regenerative medicine, the field of tissue engineering (which typically combines biodegradable scaffolds, (stem) cells, and bioactive signals such as growth factors) has created new possibilities to produce implantable tissues *ex vivo*. After several decades of development, however, the simple combination of cells and biomaterials is still far from leading to successful tissue reconstruction. One limitation that restricts its widespread application is the basic design and preparation of conventional biomaterials, which generally fail to stimulate the human body's inherent capability of self-healing. Therefore, there is a strong demand for a new generation of

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biomaterials of enhanced complexity and functionality that not only provide architectural support for cell/tissue growth but also, more importantly, mimic the complex interactions between cells and the extracellular matrix (ECM) to orchestrate cellular behavior and induce functional tissue regeneration.

Micro- and nanospheres have drawn increasing interest in the field of regenerative medicine during the past decade, which can be used as functional components in novel biomaterials of improved functionality. Microspheres (here defined as ranging in diameter from 1 to 1000 μm) have been investigated for biomedical applications for several decades, but studies on the application of nanospheres (defined here as spheres with diameters between 10 and 1000 nm) in tissue engineering only emerged in the past 10 years, thus reflecting the rapid development of micro- and nanotechnology in the field of tissue engineering. With respect to the use of micro- and nanospheres for tissue engineering and regeneration, four major strategies can be discerned to introduce these spheres as functional components to improve the performance of conventional bulk biomaterials. First, micro- and nanospheres can be used for controlled delivery of therapeutics, chemical agents, and even cells; the spheres act as delivery vehicles because of their inherently small size and corresponding large specific surface area. The size and morphology of micro- and nanospheres facilitate a high drug-loading efficiency, a quick response to stimuli from the surrounding environment, a high reactivity toward surrounding tissues *in vivo*, and a high diffusibility and mobility of drug-loaded particles.[4–12] Specifically, by incorporating spheres loaded with biomolecules of interest into a continuous matrix, classical scaffolding biomaterials can release signaling molecules without compromising the properties of the bulk scaffold.[4–6,13] Second, micro- and nanospheres can be used to alter the mechanical performance of monolithic scaffolds either by acting as porogens to create porosity in otherwise dense scaffolds[14] or as reinforcement phase to improve the mechanical strength of weak matrices.[15,16] Third, by creating a protective microenvironment inside the spheres, micro- and nanospheres can be used as compartmentalized microscopic bioreactors for dedicated biochemical processes.[17] For instance, micro- and nanospheres can be used to induce formation of biominerals and subsequently trigger mineralization of surrounding hydrogels to form self-hardening biomaterials. This strategy is inspired by the process of endochondral bone formation, in which matrix vesicles function as microcapsules to create a compartmentalized environment for the nucleation and formation of bone mineral.[18] Fourth, micro- and nanospheres can serve as building blocks to establish macroscopic, shape-specific colloidal systems that can be used as injectable or moldable scaffolds for tissue engineering. This bottom-up strategy for design and manufacture of biomaterials has recently been advocated as a promising method to develop materials with a highly defined structure and precisely controlled properties.[19,20]

This chapter focuses on the most recent advances in research on micro- and nanospheres aiming at improvement of the functionality and clinical efficacy of traditional scaffolds for soft and hard tissue engineering.

9.2 MATERIALS CLASSIFICATION OF MICRO- AND NANOSPHERES

With regard to applications in tissue engineering, micro- and nanospheres should fulfill the basic requirements that apply to virtually all biomaterials, including biocompatibility, biodegradability, nontoxicity of degradation products, and ease of processing. In general, micro- and nanospheres can be categorized into polymeric, ceramic, and composite materials.

Polymeric micro- and nanospheres have been studied most extensively for applications in controlled delivery and tissue engineering since the 1970s, when polymeric microspheres were initially introduced as drug delivery systems. The advantages of polymers over inorganic biomaterials include the ease of processing, high degree of control over the physicochemical properties (such as biodegradability), and ease of functionalization and modification. Depending on their origin, polymeric micro- and nanospheres can be classified as either natural or synthetic polymers, both of which have their specific pros and cons.

Natural polymers are an important class of biomaterials in tissue regeneration basically because of their intrinsic biocompatibility and biodegradability. Because they are derived from natural organisms, natural polymers are generally characterized by an excellent biocompatibility, biodegradability, a negligible immunogenicity, an abundant presence of side groups allowing for further chemical functionalization, and the presence of cell-recognition motifs (in the case of protein-based polymers, e.g., collagen, gelatin and fibrin).[12,21–23] Micro- and nanospheres made of natural polymers can be prepared by simple emulsion techniques in which spheres of variable properties (size and morphology) can be obtained by tailoring the emulsification process.[24,25] The resultant micro- and nanospheres are widely accepted as desirable vehicles for drug or biomolecule delivery because of the gentle gelling conditions that facilitate encapsulation of biomolecules and cells, controllable release kinetics by fine-tuning the degradation of carriers, and ease of functionalization.[26–29] Despite the favorable properties, several critical concerns about natural polymers include (1) poor mechanical properties that hamper applications under load-bearing conditions,[21,22] (2) immunogenicity or the risk for disease transfer for polymers extracted from allogeneic or heterogenous sources,[30] and (3) poor control over physicochemical characteristics (e.g., molecular weight).

On the other hand, synthetic polymers, such as poly(lactic acid) (PLA) and poly (lactic-*co*-glycolic acid) (PLGA), are also of considerable importance for regenerative medicine applications owing to their biocompatibility, biodegradability, well-defined physicochemical properties (e.g., molecular weight), defined mechanical properties, ease of fabrication and modification, and the absence of the possibility to transfer diseases. Micro- and nanospheres composed of synthetic polymers have been widely investigated as delivery vehicles for therapeutic agents[31–33] and building blocks for tissue engineering scaffolds.[34,35] However, drawbacks related to the use of micro- and nanospheres made of synthetic polymers include the acidic degradation products, hydrophobicity, degradation by autocatalysis, and low drug-loading efficacy.[36]

To develop biomaterials of enhanced physicochemical and biological properties, composite materials have gained considerable attention for tissue engineering

applications over the past decades. By incorporating different components, composites combine the advantages but eliminate the drawbacks of each component, resulting in improved functionality and complexity. Representative examples include inorganic–organic composites for bone reconstruction, which typically combine biodegradable polymers with bioactive ceramics, resulting into materials that improve the biological performance of polymers as well as provide bioceramics with the ease of processing and controllable degradation. Composite micro- and nanospheres have been fabricated by incorporating bioceramics (e.g., calcium phosphates (CaPs)) with biopolymers (e.g., gelatin, PLGA),[37–44] which displayed improved biological and physicochemical properties that include enhanced hydrophilicity (compared with pure PLGA microspheres),[45] higher drug-loading efficiency,[46] improved cytocompatibility,[45] reduced biodegradation and drug release rates,[38] and strongly upregulated *in vitro* calcifying capability.[38]

9.3 APPLICATIONS OF MICRO- AND NANOSPHERES IN TISSUE ENGINEERING

9.3.1 Micro- and Nanospheres as Delivery Vehicles

9.3.1.1 Delivery of Biomolecules A critical challenge in tissue engineering is to control the delivery of signaling biomolecules at the treatment sites to provide instructive signals that regulate cell behavior and facilitate tissue regeneration. To this end, complex and sophisticated delivery systems are required that allow for sustained presence of therapeutic components at target tissues at the proper time. Micro- and nanospheres have been studied most extensively for controlled delivery of biomolecules owing to their inherently small size and corresponding large specific surface area, high drug-loading efficiency, high reactivity toward surrounding tissues *in vivo*, and high diffusibility and mobility of drug-loaded particles (Fig. 9.1).[4–8,13]

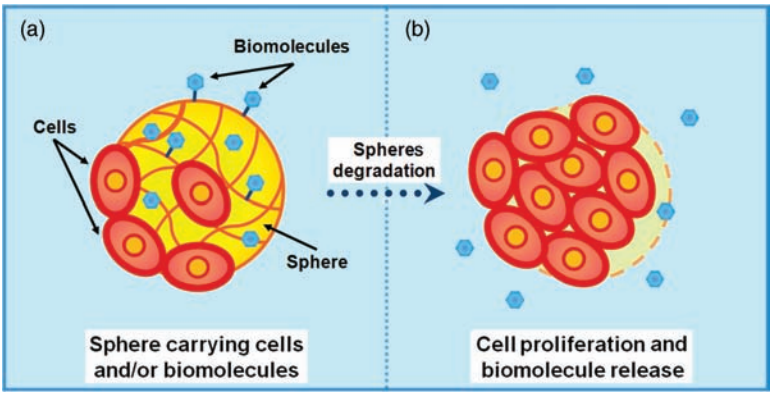

FIGURE 9.1 The use of degradable microspheres as vehicles for biomolecule or cell delivery (a), which can lead to subsequent cell proliferation and biomolecule release with the degradation of carriers (b).

The basic strategy to use micro- and nanospheres as carriers to load biomolecules is by simply adsorbing onto the particle surface and subsequently releasing their payload *in vivo* by desorption, diffusion, or carrier degradation depending on the chemical composition and geometry of the spherical vehicle. The poor control over the release of biomolecules is the main drawback that has limited widespread application of this method. Therefore, new methods have been developed that either (1) physically entrap biomolecules in the carrier matrix or (2) chemically immobilize biomolecules to the polymer backbone. Subsequently, release can be obtained upon degradation of the spheres or hydrolytic or enzymatic cleavage of the chemical bond between the carrier and the biomolecule.[47–49] More specifically, microencapsulation has been developed as a promising strategy for controlled delivery of biomolecules by physically compartmentalizing biomolecules into the hollow interior of micro- and nanospheres, thus protecting labile biomolecules from denaturation by harsh environmental factors. Release profiles of encapsulated biomolecules normally display sustained-release kinetics favorable for long-term delivery compared with molecules adsorbed onto surface of carriers.[50,51] Nkansah et al. prepared PLGA micro- and nanospheres with ciliary neurotrophic factor encapsulated inside the spheres by emulsification, which can be used as delivery vehicles for growth factor delivery without compromising their bioactivity.[52] Alternatively, biomolecules loaded into spherical carriers by chemical immobilization techniques normally show prolonged retention at the delivery site with a target-specific manner.[4,53–55]

Regarding the clinical application of drug-loaded micro- or nanospheres, one simple delivery strategy involves incorporation of micro- and nanospheres loaded with therapeutic components into a continuous matrix of monolithic scaffolds, thus prolonging the retention of biomolecules at the implantation site but also providing bulk scaffolds with enhanced features for controlled and sustained release of drug or proteins.[4–6,56–58] Especially for controlled delivery of biomolecules, simple incorporation of biomolecules into bulk materials probably leads to their denaturation or deactivation caused by exposure to harsh processing conditions, hydrophobic surfaces of polymers, or acidic degradation products.[58] However, incorporating biomolecule-loaded spheres into polymer scaffolds was found to be of more efficiency with a reduced initial burst release followed by a slow, sustained release of biomolecules compared with a release profile using microsphere-free scaffolds.[59]

Moreover, programmed delivery of multiple biomolecules with precise spatiotemporal control over the distribution of biomolecules throughout scaffolding materials or sequential release of various molecules can be achieved by incorporating micro- and nanospheres as delivery system into classical scaffolds.[33,60] Temporal control over biomolecule delivery can be realized by using various microsphere or nanosphere populations for different biomolecules. In doing so, distinct release profiles of each components can be obtained by tailoring the physicochemical properties of each spheres and the corresponding release behavior, resulting in temporally controlled drug delivery.[60] For example, sequential release of dual growth factors was obtained by combining both poly(4-vinylpyridine) and alginate microspheres to load and release bone morphogenetic protein 2 (BMP-2) and BMP-7 independently.[61] Furthermore, spatial control of signaling molecules is of growing

interest for engineering of many tissues such as nervous[62] and osteochondral[63] tissues. In these applications, a gradient distribution of bioactive signals is established to induce concentration-dependent cell responses.[64,65] To this end, Wang et al. developed scaffolds containing reverse concentration gradients of two growth factors (BMP-2 and insulin-like growth factor I (IGF-I)) through polymer scaffolds for osteochondral reconstruction by introducing silk and PLGA microspheres as carriers for each growth factor.[66] In that way, human mesenchymal stem cells (MSCs) were stimulated to differentiate into osteoblasts and chondrocytes, respectively.

9.3.1.2 Delivery of Cells Besides delivery of therapeutic or biochemical components, biodegradable and cytocompatible microspheres can also serve as cell delivery vehicles to improve the biological performance of tissue engineering constructs (Fig. 9.1) or to construct microscopic three-dimensional (3D) tissue equivalents that mimic the native tissue structure. In contrast to conventional hydrogel-based cell encapsulation approaches that normally lead to cell death because of limited cell adhesion, migration, and communication,[67] the introduction of microspheres as cell carriers into hydrogels not only provides cellular focal adhesions (in case of arginine-glycine-aspartic acid (RGD)-containing polymers) but also facilitates cells to overcome gel restriction and fully spread out into their natural morphology.[67–70] Wang et al. proposed an injectable hydrogel scaffold based on encapsulation of cell-laden gelatin microspheres into a continuous matrix of agarose hydrogel, which exhibited strong potential for cell conveyance and regeneration of bone and other tissues.[67,69,70] Considering the above mentioned approach as traditional scaffold-based "top-down" strategy to create cellularized constructs, "bottom-up" tissue fabrication methods using cell-laden microspheres as building blocks are potentially more powerful tools to construct 3D hybrid constructs comprising both cells and biomaterials.[71,72] Matsunaga et al. recently developed a method for rapid construction of macroscopic 3D constructs using a large number of monodisperse cell-laden collagen microspheres with monodispersity to assemble into uniform and shape-specific tissues.[71] Similarly, Pautot et al. proposed a colloidal superstructure based on monodisperse silica microspheres for 3D neuronal network formation. These microsphere-based bottom-up strategies showed many advantages, including (1) a large surface area provided by microspheres for cell adhesion and further functionalization; (2) abundant interparticle cavities, allowing for nutrient exchange *in vitro* and *in vivo*; and (3) ease of manipulation and transportation of colloidal microspheres.[71,73]

9.3.2 Micro- and Nanospheres as Functional Components to Modify Mechanical Properties of Scaffolds

9.3.2.1 Use of Micro- and Nanospheres as Porogens By embedding microspheres into the continuous matrix of bulk materials, spheres can serve as porogen to introduce porosity into otherwise dense biomaterials (Fig. 9.2). A typical example of this strategy is the incorporation of microspheres into calcium phosphate cements (CPCs), which exhibit slow degradation rates *in vivo* and consequently a lack of

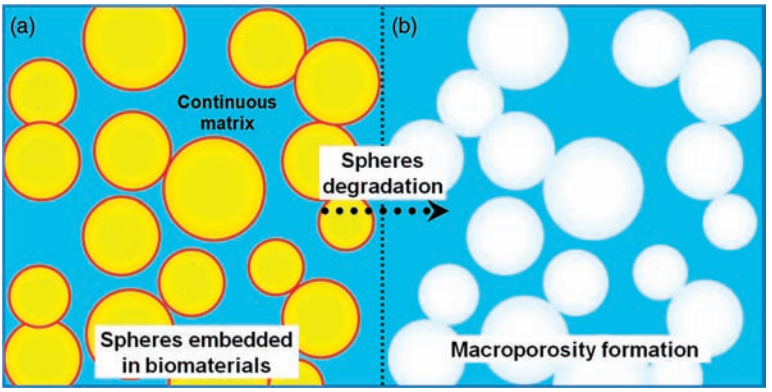

FIGURE 9.2 The use of spheres as porogens to introduce macroporosity to otherwise dense biomaterials by embedding spheres into the continuous matrix of bulk materials (a), thus creating porosity with the degradation of the spheres (b).

macroporosity and new bone ingrowth. By introducing degradable polymeric microspheres (made of, e.g., PLGA[14] and gelatin[74,75]), macroporosity can be formed introduced upon degradation of microspheres, which can subsequently create space for cell and tissue ingrowth and accelerate the resorption of CPCs.[14] Additionally, this strategy can make injectable CPCs suitable for cell encapsulation and biomolecule delivery to upregulate the extend of bone regeneration even further.[76,77]

Another method to use micro- and nanospheres as porogens to create porous structures is a technique referred to as *colloidal crystal templating*. This technique involves the formation of a body of closely packed monodisperse spheres, after which the interstitial space is filled with a solidifying fluid precursor followed by removal of the template to obtain a porous inverse replica.[78] The resultant so-called inverted colloidal crystal (ICC) scaffolds with highly ordered macroporosity displayed significant advantages compared with traditional processing techniques, including a tightly controlled pore size (ranging from nanometer to micrometer scale); a well-defined periodic hierarchical porous structure with high interconnectivity; a highly accessible surface and large pore size; and the possibility to include pores of different sizes, allowing for selective uptake of small or large biomolecules.[79–81] Moreover, scaffolds produced using this technique are characterized by a uniform distribution of cells throughout the porous matrix, thus creating a highly standardized microenvironment for cell encapsulation.[82]

9.3.2.2 Use of Micro- and Nanospheres as Reinforcement Components Micro- and nanospheres can be incorporated into continuous matrices to provide additional mechanical support for traditional biomaterials by serving as reinforcement components[15] or cross-linking agents.[16] Ceramic micro- or nanoparticles are favorable candidates in the reinforcement phase to be incorporated into polymer matrices because of their intrinsically higher mechanical strength. For instance, β-tricalcium phosphate (β-TCP) MSCs were combined with alginate hydrogels to form an

injectable 3D constructs that can encapsulate MSCs in which β-TCP microspheres of high stiffness reinforced the mechanical strength of the alginate matrix.[15] On the other hand, micro- and nanospheres embedded into the continuous phase of biomaterials can also act as cross-linking anchors to form direct bridges between micro- and nanospheres with the surrounding network or function as delivery vehicles that encapsulate cross-linking agents and subsequently release them to trigger cross-linking of the surrounding polymer phase. For instance, positively charged PLA microspheres were embedded in an anionic polymer phase of hyaluronic acid to induce gelation of hyaluronic acid by forming polyion complexes without introducing cross-linking chemicals that can be cytotoxic.[16] Moreover, in the design of so-called self-healing biomaterials, microspheres can be used as microcapsules containing an active healing agent dispersed in a polymer matrix. When a propagating crack encounters a microcapsule and causes its rupture, the healing agent is released to initiate a repolymerization process, thus filling the crack area.[83] This approach of using microspheres in designing self-healing biomaterials is an excitingly new area that can be of great benefit in the development of novel biomaterials.

9.3.3 Micro- and Nanospheres as Microreactors

Hollow micro- and nanospheres (microcapsules) have been investigated recently for their potential to serve as microscopic bioreactors for dedicated biochemical processes in biomedical applications.[17,84,85] Candidates for this purpose include polymeric capsules, liposomes, polymersomes, and so on that can (1) create a inner compartment capable of efficient entrapment of components of interest; (2) provide a sufficiently robust and stable shell, allowing for selective diffusion of substrate components or reaction products into or out of the capsules; and (3) introduce no harmful effect to native cells and tissues.[85,86]

A representative example of using microcapsules for biomedical applications is the controlled formation of biominerals in defined compartments. This strategy is inspired by the process of endochondral bone formation that uses nanosized matrix vesicles as initial sites of biomineralization.[17,18] To this end, Michel et al. developed an approach using liposomes encapsulated with calcium ions and alkaline phosphatase (the enzyme that releases inorganic phosphate ions from organic phosphate esters *in vivo*) to induce CaP crystals formation under well-controlled conditions (Fig. 9.3).[87] Similarly, Pederson et al. developed calcium- and phosphate-loaded liposomes in combination with collagen hydrogels, which facilitated *in situ* formation of CaP crystals and subsequent mineralization of hydrogels, and finally formed self-hardening biomaterials that can be applied as injectable, self-gelling formulations for bone regeneration.[17] Another biomimetic approach for inducing biomineral formation inside polyelectrolyte capsules was developed by Antipov et al.[88] based on urease-catalyzed precipitation of carbonate in the capsule interior. By suspending urease-loaded capsules in aqueous solutions containing $CaCl_2$ and urea, $CaCO_3$ mineralization was triggered because of the impermeability of urease macromolecules inside the capsules, but high permeability of small urea molecules and Ca^{2+} through the capsule wall allowed for precipitation of inorganic crystals. These

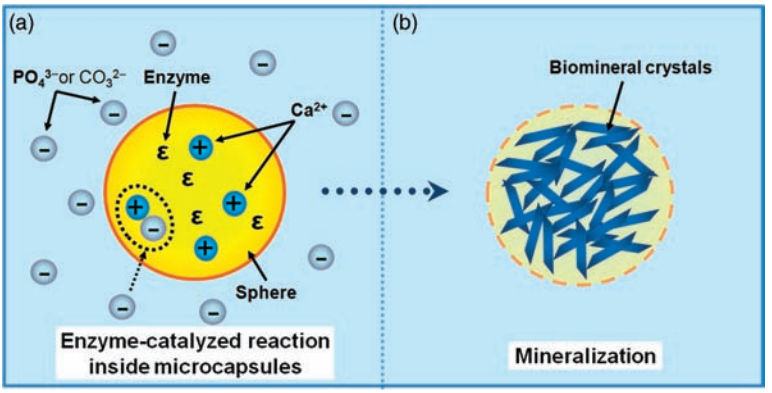

FIGURE 9.3 The use of spheres as microscopic bioreactors for controlled formation of biominerals. Enzyme-catalyzed reaction can be triggered in a defined compartment (a), thus facilitating the nucleation and formation of biominerals crystals inside microcapsules (b).

dedicated biomimetic strategies for biomineral formation using defined microcapsules provide a promising pathway for the development of hybrid organic–inorganic biomaterials that can be used for tissue engineering and regeneration.

9.3.4 Micro- and Nanospheres as Building Blocks

Recently, "bottom-up" strategies for the design of novel biomaterials have been advocated as a new paradigm for development of a new generation of tissue engineering scaffolds. One example of such a bottom-up strategy uses micro- or nanoscale particles as building blocks to (self-)assemble into macroscopic structures. Micro- and nanospheres are obvious candidates as structural building blocks for such applications, in which integrated structures can be formed by either random packing or directed self-assembly (Fig. 9.4). As opposed to traditional monolithic implants, these sphere-based scaffolds display several advantages for tissue engineering such as a precise control over the physicochemical characteristics of scaffolds (e.g., degradation rate) by fine-tuning the specific structural units,[89] the ease of encapsulation of therapeutic[90] or biochemical[29,91] components, and desirable clinical handling properties (i.e., injectability and moldability).[72,92]

The most basic strategy to create scaffolds composed of micro- and nanospheres is by randomly packing the spherical building blocks together, which normally results in a moderately organized 3D structure of poor cohesion.[72] Polymeric micro- and nanospheres (e.g., gelatin,[20] chitosan,[93,94] alginate,[95] and PLGA[34] micro- and nanospheres) have been used to build up scaffolds by simply packing them together, thus forming injectable formulations that can be used as defect fillers for tissue regeneration. However, one critical concern of this strategy for *in vivo* applications is the poor integrity of the spheres because of the lack of interparticle interactions, which could potentially lead to poor mechanical stability and high flowability of the scaffolds[96] and ultimately detrimental side effects to the surrounding tissues caused

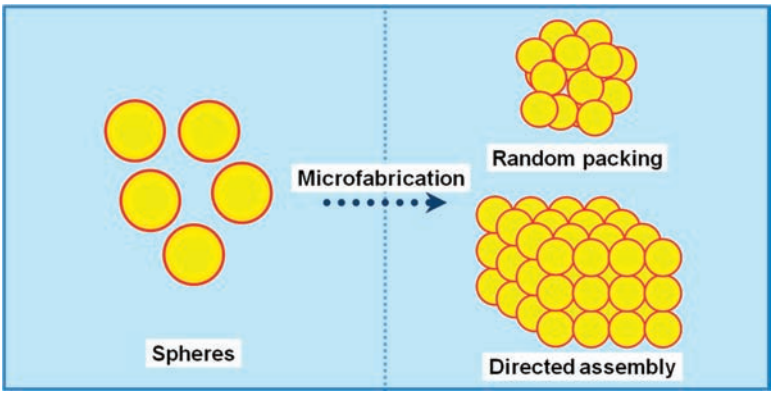

FIGURE 9.4 The use of micro- and nanospheres as building blocks to assemble into macroscopic structures by either random packing or directed assembly of the spheres.

by the individual particles migrating from the treatment sites.[97] To address this problem, efforts have been made to increase the cohesion of micro- and nanosphere-based formulations at the implantation sites (e.g., by using glues or additional interparticle cross-linkers).[97,98] Alternatively, sintered microsphere-based scaffolds were developed by fusing densely packed PLGA[99] or chitosan[100] microspheres together by thermal treatments. These scaffolds exhibited tailorable morphological and compositional properties of the scaffolds,[99] controllable biomolecules release profiles,[31,63] *in vitro* and *in vivo* biocompatibility,[101,102] and a degree of degradability suitable for tissue engineering applications.[103]

Directed assembly of micro- and nanospheres into cohesive macroscopic constructs has recently been advocated as a more sophisticated strategy to design particle-based scaffolds by maximizing interparticle interactions (e.g., electrostatic, magnetic, or hydrophobic interactions) as driving forces to induce self-assembly of micro- and nanospheres. Specifically, colloidal gels have been developed recently based on self-assembly of micro- or nanospheres directed by either electrostatic[35,104–106] or hydrophobic[107] interactions, which showed desired structural integrity and mechanical stability in physiological conditions,[105] excellent injectability and moldability, and capability of self-recovery after network destruction because of the reversible physical cross-linking features that characterize these self-assembling systems.[35,105,107] These physical gels showed great potential to be used as injectable fillers for regenerative medicine by using minimally invasive surgery. For example, Wang et al. prepared injectable colloidal gels made of oppositely charged, dexamethasone-loaded PLGA nanospheres, which displayed a nearly zero-order drug release profile *in vitro* and induced bone formation *in vivo*.[108] Similar to electrostatic and hydrophobic interactions, magnetic force can also be used as a powerful tool to trigger self-assembly of micro- or nanoscale building blocks to generate integrated structures as tissue engineering scaffolds.[109,110] Interestingly, instead of using magnetic micro- and nanospheres as building blocks, Ito et al. recently developed magnetic nanosphere-labeled cells as structural units to form a

scaffold-free, cell-patterned structure.[111–113] This so-called magnetic force-based tissue engineering strategy showed potential to construct 3D cellularized tissues even without using monolithic scaffolds.

9.4 CONCLUSIONS

Micro- and nanospheres have evolved as powerful tools in the design of novel biomaterials for controlled delivery and tissue engineering and regeneration. Strategies using micro- and nanospheres display several advantages compared with conventional monolithic biomaterials, such as (1) an improved performance in controlled and sustained delivery of therapeutic agents, signaling biomolecules, and even (stem) cells; (2) improved structural or mechanical properties of bulk scaffold by using spheres as porogens or reinforcement phase to introduce porosity or improve mechanical strength; (3) upregulated control over dedicated biochemical processes by using micro- and nanospheres as compartmentalized microreactors; and (4) the possibility to prepare self-assembling colloidal systems that can be used as injectable or moldable formulations to be applied using minimally invasive surgery.

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10

MICRO- AND NANOTECHNOLOGIES TO ENGINEER BONE REGENERATION

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10.1 INTRODUCTION

The prevalence of bone fractures in the United States is a major health care concern with more than 1 million new incidences every year.[1] Non-union fractures occur when the broken bone loses the ability to self-heal and can be classified as atrophic, lacking healthy cells or vasculature; hypertrophic, containing healthy cells and vasculature and able to heal when stable; or oligotrophic, a transition stage between the previous two fracture types.[2] Bone loss in non-union fractures requires specialized treatment strategies such as the use of a set and cast or in some cases surgery in which the fracture is stabilized by a pin or plate.[3,4] These methods are accompanied by medications to alleviate pain and delay healing times depending on the site of fracture.[4] In more severe cases, when damaged bone is either removed or lost, bone implants play a vital role in tissue regeneration and healing.

Bone implants can be autografts or allografts. Autograft bone implants are patients' own bone used for grafting procedures to replace damaged bone tissue. Although autografts are highly successful because of low risk of immunological rejections, they require extraction of bone from a healthy part of the individual, leading to deterioration of the donor site, pain, and risk of infection. Allografts, bone implants harvested from other individuals, can be rejected by the host immune system. Apart from immune rejection, they have a high risk of infection with the

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additional possibility of acquiring fatal diseases from the donor.[5] Recently, artificial bone grafts have been developed that eliminate the risks associated with autografts and allografts. They can be formulated specifically for every patient depending on the fracture site and host immune response. Although artificial bone grafts have improved the quality of bone implants, concerns of biocompatibility, biodegradability, and reduced mechanical properties are associated upon implantation in humans.[5] Bone tissue engineering, incorporating the knowledge of biological systems, and engineering, has the potential to address the aforementioned concerns associated with the use of articifial bone implants in humans. One of the most widely used strategies in bone tissue engineering is the use of scaffolds for temporary structural support. Scaffolds are porous biomaterials and play a central role in tissue engineering approaches by guiding cell proliferation and assisting the exchange of nutrients and waste.

The goal of contemporary bone tissue engineering research is to formulate a scaffold that mimics the mechanical properties of native tissue. The mechanical properties of human bones have been extensively studied and characterized.[6] The compression modulus and compression strength of human cortical bone have been reported as 17–20 GPa and 106–133 MPa, respectively.[7] The flexural modulus of bone, as reported, is 15.5 GPa, and the flexural strength is 180 MPa.[8]

Nanoparticles have been incorporated into tissue engineering scaffolds to increase their mechanical properties.[6,9–15] A widely accepted definition of a nanoparticle/nanofiber is a material having size less than 100 nm in one dimension. Nanomaterials interact with the scaffold matrix by weak Van der Waals interactions or hydrogen bonds or may covalently bind to the polymer. The nanoparticle-incorporated scaffolds provide mechanical support and the microenvironment necessary for cells to differentiate and mature. The surface properties of nanoparticle-incorporated scaffolds allow better interaction of cells with proteins, creating an extracellular matrix (ECM), which in turn facilitates cell growth and tissue regeneration.[1] To increase the mechanical properties of nanomaterial-incorporated scaffolds, covalent bonds between nanoparticles or nanofibers and polymer chains are highly desired, permitting efficient mechanical load transfer and formulation of tougher nanocomposites.[16]

In this chapter, selected published articles pertaining to micro- and nanotechnologies for bone tissue engineering are reviewed with a focus on development of scaffolds.

10.2 NANO-HYDROXYAPATITE REINFORCED SCAFFOLDS

Hydroxyapatite (HAp or $Ca_{10}(PO_4)_6OH_2$),[17] a ceramic widely used in bone tissue engineering applications, reduces stress shielding and increases the biocompatibility of the implant.[18,19] HAp is responsible for the release of calcium (Ca) and phosphorus (P) ions, which are used as substrates during bone remodeling.[18] Recent studies have shown that nanohydroxyapatite (nHAp) has improved protein adsorption capabilities compared with macro- and micro-HAp.[19–21]

In bone, HAp exists as needle-shaped crystals with a size distribution of 20–60 nm, whereas nHAp can be found in rods, fiber, or particulate form.[18,22]

The morphology of nHAp is governed by the mode of synthesis, which may be mechanochemical based, in which the material is created by a heterogeneous reaction between solids dependent on the perturbation of surface-bonded species;[23] may be combustion based, in which calcium carbonate is subjected to a temperature of 900 °C, resulting in CO_2 release and the formation of CaO, which yields HAp in a phosphate solution;[24] or may use wet chemistry techniques involving direct precipitation via sol-gel synthesis.[25] Precipitation synthesis of HAp crystals involves the use of modifiers, chemical compounds that may influence the morphology of HAp crystals. Some of these modifiers include citric acid, amino acids, and ethylenediamine-tetra-acetic acid (EDTA).[26] The morphology of HAp crystals can also be governed by changes in pH with a pH greater or equal to 7 resulting in the formation of nHAp crystals and a pH below 7 leading to the formation of microcrystals.[26]

HAp crystals are brittle, and scaffolds formulated with HAp in matrix have significantly low mechanical properties, making them unsuitable for load-bearing applications.[27] HAp is widely used as a bone void filler.[28] Owing to its morphology, nHAp possesses greater surface area compared to micro-HAp, which can be exploited to yield a dense packaging of nHAp in the scaffold matrix.[26] A dense packaging leads to significant enhancements in the mechanical properties, which in conjunction with the similarity of nHAp to native tissues (with respect to size and chemistry) make nHAp a favorable material for bone tissue engineering.[18] However, one of the prime reasons for the use of nHAp is to increase the mechanical properties of the polymer matrix.[9,17–19,27,29–32] This is analogous to natural bone, a composite of apatite crystals within a collagen matrix.[13]

nHAp and chitosan nanocomposites have been studied as scaffolds for bone tissue engineering applications.[13,33] Polymers such as poly(L-lactic acid) (PLLA),[34]poly (ester urethane) (PU),[19] poly(vinyl alcohol) (PVA),[17] and poly(D,L-lactic-*co*-glycolic acid) (PLGA) have been widely investigated as a scaffolding matrices, containing HAp crystals.[27] Uniform dispersion of nHAp within the polymer matrix is of utmost importance because unequal distribution can lead to voids after bone formation (Fig. 10.1).[9]

HAp-incorporated scaffolds can be formed by various techniques such as electrospinning,[13,15,31] gas foaming and particulate leaching,[27] salt leaching–phase inversion[19] followed by mixing in an acidic environment, and lyophilizing.[33] Electrospinning is an extensively used technique for the production of polymer fibers. A well-dispersed nanomaterial–polymer composite is an essential prerequisite before electrospinning scaffolds to prevent the agglomeration of nanomaterials.[13] Electrospinning involves the exposure of nanomaterial–polymer solution to an electric field within a capillary tube. When the electric field overcomes the surface tension of the material, a jet of polymer solution is released from the capillary. The polymer solution undergoes stretching as the collector is grounded, resulting in the formation of fine electrospun fibers.[35] Gas foaming and particulate leaching involve exposure of salt containing polymer matrix to a high-pressure gas before the salt leaching step.[27] The salt leaching–phase inversion technique involves the mixing of nHAp–polymer solution with a porogen, followed by exposure to air to evaporate solvents, and washing steps to remove the porogen (Fig. 10.2).[19]

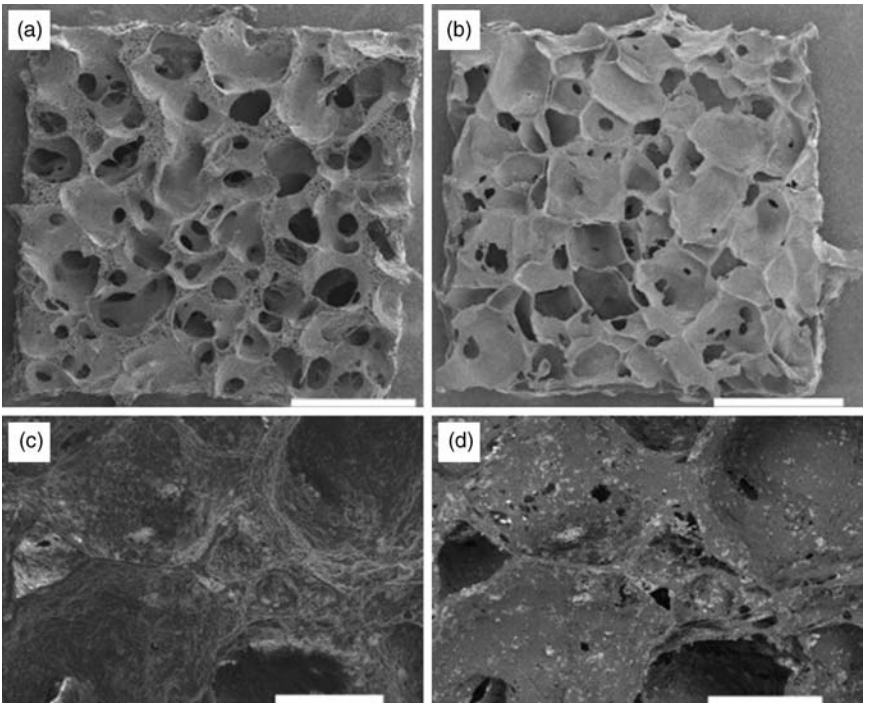


FIGURE 10.1 Scanning electron microscope micrographs of (a) polyurethane (PU) and (b) nHAp/PU scaffold. Note that nHAp scaffolds exhibit a decreased microporosity compared with the control. Scale bars: 1 mm. (c and d) Higher magnification images of (a) and (b). Scale bars: 100 μm. Adapted from Ref. [29] © Elsevier 2010.

The toxicity of nHAp sponges, assessed by a trypan blue viability assay, confirmed the absence of necrosis of human bone marrow stromal cells (HBM) after 14 days of exposure.[36] Another study reported the cytotoxicity of varying amounts of HAp (0, 10, 20, 30, 40 or 50 wt%) added to PLLA on rat mesenchymal stem cells. Cellular viability assessed by alamarBlue assay after 1 and 2 weeks of exposure indicated the nontoxic nature of materials.[18] Two studies on the toxicity of nHAp composites, one with chitosan and nanosilver (nano-Ag) containing 1:1 ratio of nHAP:chitosan, and the other with copper (Cu) and polyethylene glycol 400 (PEG 400), reported the absence of toxicity of the composites on rat osteoprogenitor cells, assessed by MTT assays after 24 h incubation.[33,37]

In addition to nHAp, nano-Ag and Cu have also been dispersed in the polymer matrix. The addition of nano-Ag or Cu imparts antibacterial properties to the polymer material. Specifically, nano-Ag imparts antibacterial properties against both gram-positive and -negative bacteria, but the addition of Cu preferentially inhibits the growth of gram-positive bacteria.[33,37] nHAp–multi-walled carbon nanotube (MWCNT) composites have also been formulated for bone tissue engineering applications. Addition of 7 vol.% of MWCNTs increased the biaxial strength and

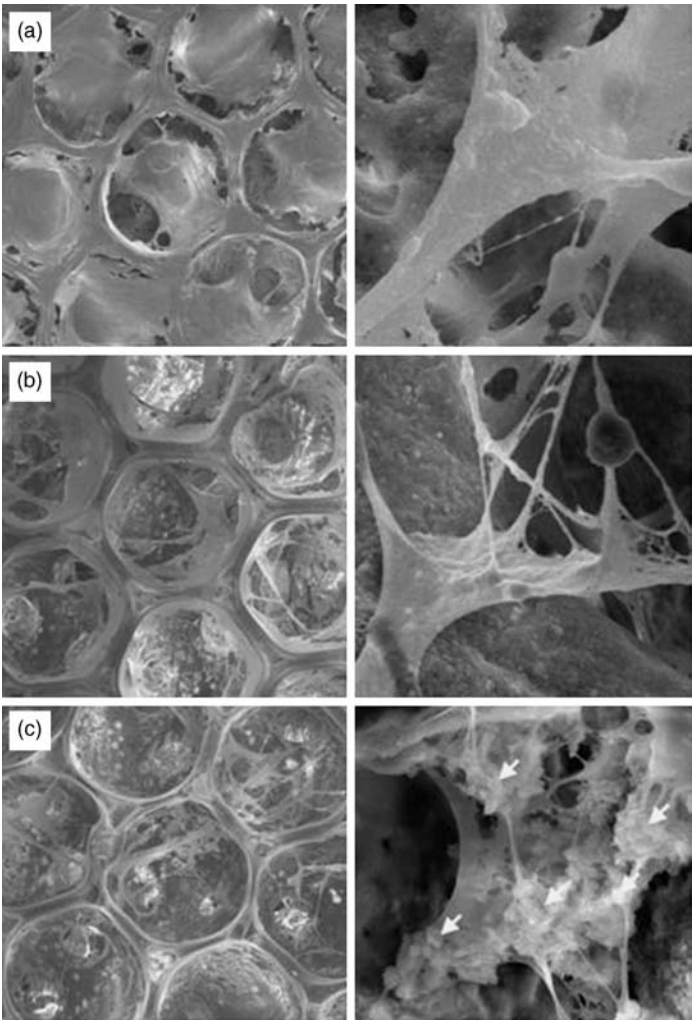


FIGURE 10.2 Scanning electron microscope micrographs of cells cultured on (a) PLGA scaffolds, (b) PLGA-HAp, and (c) Ap-coated PLGA-HAp scaffolds for 28 days. Large numbers of nodules such as minerals (indicated as *arrows*) were observed on the surface of Ap-coated PLGA-HAp scaffolds. Adapted from Ref. [9] © ACS 2010.

toughness of the composites by 28 and 50%, respectively. Further increase in MWCNT loading concentration decreased the strength and toughness of the composites due to formation of MWCNT aggregates resulting in weakness at the interface between nHAp and MWCNTs (Fig. 10.3).[38]

nHAp–polymeric scaffolds seeded with marrow-derived (40 and 200 μg/10 mesenchymal stromal cells (MSCs) have been reported to stimulate the growth of MSCs at concentrations less than 20 μg/10^4 cells. However, at higher

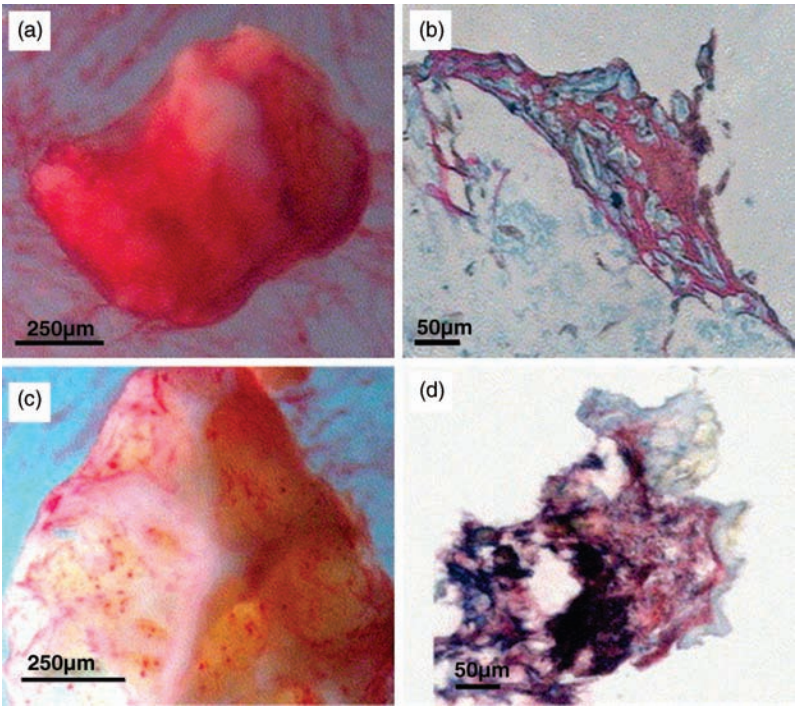


FIGURE 10.3 Photomicrographs of HBM (human bone marrow) cells grown on HAp-alanine and HAp-dextran spongelike scaffolds. (a and c) Expression of alkaline phosphatase (red staining) and (b and d) collagen production (Sirius red and Alcian blue staining). Adapted from Ref. [36] © Elsevier 2005.

concentrations 40 and 200 μg/104 cells, nHAp inhibited cell growth. Moreover, differentiation of cells occurred when the cells and nHAp were in osteogenic media coupled with an inhibitor of mineralization of cells.[20] In another study, nHAp–PLGA scaffolds have been reported to stimulate the osteogenic differentiation of preosteoblast cells after 6 weeks in culture. Micro computed tomography analysis revealed the even distribution of secreted minerals throughout the scaffold.[9] When included in cyclic acetal hydrogels, nHAp particles enhanced the differentiation of MSCs into osteoblasts, observed by an increased osteogenic gene expression (bone morphogenic protein -2, alkaline phosphatase, and osteocalcin).[21]

10.3 BIODEGRADABLE POLYMERIC SCAFFOLDS AND NANOCOMPOSITES

Synthetic polymers such as poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) and their copolymer PLGA have been investigated for bone tissue engineering applications. PLGA is a biocompatible, biodegradable polymer with enhanced

mechanical properties compared to PLA and PGA. The Food and Drug Administration (FDA) has approved PLGA for clinical and basic research in drug delivery, vaccination, cardiovascular diseases, and tissue engineering applications.

PLGA synthesis involving ring-opening co-polymerization of PLA and PGA uses tin (II) 2-ethylhexanoate, tin (II) alkoxides, or aluminum isopropoxide as catalysts.[11] During the reaction, the monomers PLA and PGA are linked together by ester linkages. Depending on the ratio of monomers present at the onset of the reaction, various forms of PLGA can be synthesized. PLGA 75:25 contains 75% PLA and 25% PGA. Similarly, PLGA 65:35, PLGA 50:50, and PLGA 85:15 are also commercially available. PLGA is hydrolyzed to its monomers (PLA and PGA) in the presence of water. Because these monomers are the byproducts of various metabolic pathways in the body, they can easily be metabolized and degraded without any complications.[39]

A challenge in bone tissue engineering is to design a scaffold that mimics the mechanical properties of natural bone ECM. Polymers by themselves do not have the mechanical properties comparable to native bone tissue. Therefore, nanomaterials have been used as reinforcing agents to improve the mechanical properties of polymeric composites. Some of the nanoparticles that have been incorporated into PLGA scaffolds are HAp nanoparticles, single-walled carbon nanotubes (SWCNTs), and titanium oxide microsphere.[9,11,12,40] HAp incorporated in electrospun PLGA at concentrations of 1 and 5% improved the mechanical properties of PLGA fibers. However, an increase in the concentration of HAp particles to 10 and 20% resulted in defects in the fiber, thereby decreasing the mechanical properties. In another study, HAp–PLGA nanocomposites exhibited a decreased biodegradability compared with neat PLGA, desirable for long-term stability of the scaffold.[41] Incorporating 1% SWCNT in PLGA scaffolds prepared by solvent casting increases the Young's modulus from 5.0 to 7.8 MPa.[12] Carboxylated SWCNTs in the same concentration further increased the Young's modulus, 8.3 MPa. In addition to an increase in the Young's modulus, carboxylated SWCNTs nanocomposites, also accelerated hydrolytic degradation and weight loss. Addition of pristine SWCNTs showed no significant effect on the degradation or the weight loss of the scaffolds. Nanomaterials incorporated into PLGA scaffolds can thus be used to tailor the properties of the scaffolds depending on the desired application.

Poly(propylene fumarate) (PPF), a polyester of propylene glycol and fumaric acid, is a biocompatible, biodegradable, and osteoconductive polymer widely studied for bone tissue engineering applications.[42] PPF is highly viscous and can be cross-linked with methyl methacrylate, *N*-vinyl pyrrolidinone (NVP), PPF–diacrylate, poly(ethylene glycol)–diacrylate, or itself.[42,43]

In biological systems, PPF is hydrolyzed into biocompatible fumaric acid and propylene glycol with traces of acrylic acid and poly(acrylic acid-*co*-fumaric acid). PPF and its degradation products possess low *in vitro* cytotoxicity and minimal inflammatory responses.[44,45] PPF scaffolds lack suitable mechanical properties required for bone tissue engineering applications. Nanoparticles incorporated in PPF scaffolds enhance the mechanical properties.

Recent study shows that addition of two-dimensional carbon and inorganic nanostructures such as single- and multi- walled graphene oxide nanoribbons,

graphene oxide nanoplatelets and molybdenum disulfide nanoplatelets at low loading concentrations (0.01–0.2 wt%) increase the mechanical properties (i.e. Young's modulus, compressive yield strength, flexural modulus and flexural yield strength) of PPF nanocomposites.[46]

Ultra-short single-wall carbon nanotubes (USCNTs) have been incorporated into PPF scaffolds for bone tissue engineering applications. USCNTs, homogeneously incorporated into PPF scaffolds at 0.5wt%, improved the mechanical properties of the PPF scaffold by up to 200% for the flexural and compressive properties compared with PPF alone.[47] *In vitro* cytotoxicity studies showed 100% cell viability and excellent cytocompatibility, although some adverse effects on cells were observed during degradation of the scaffold.[48] To study *in vivo* cytotoxicity, USCNT scaffolds were implanted in rabbit femoral condyles and subcutaneous pockets. Histology and histomorphometric analysis of soft and hard tissue exhibited good biocompatibility over a period of 12 weeks. Scaffolds containing USCNTs resulted in an enhanced bone regeneration compared with PPF scaffolds (Fig. 10.4).[49]

Alumoxane nanoparticles have been investigated for the fabrication of alumoxane–PPF bone tissue engineering scaffolds. Mechanical properties of alumoxane–PPF composites were characterized by compressive and flexural testing. Composites containing 1 wt% alumoxane nanoparticles exhibited more than threefold increase in flexural modulus compared with PPF controls. The enhancement of mechanical properties was attributed to the fine dispersion of alumoxane nanoparticles, and covalent bonding between nanoparticles and PPF.[16]

Degradation and biocompatibility of alumoxane–PPF nanocomposite scaffolds have been studied *in vitro* and *in vivo*. Nanocomposite scaffolds degrade significantly faster compared to PPF controls and exhibit negligible *in vitro* cytotoxicity in fibroblasts. Minimal adverse effects such as inflammation of the surrounding tissue was observed *in vivo*. It was also observed that predegraded particles increase cytotoxicity and inflammation because of their increased surface area and roughness.[50]

10.4 SILK FIBERS AND SCAFFOLDS

Silk, originating from the silkworm (*Bombyx mori*), is widely used in biomedical applications such as tissue engineering. Spider silk, not widely commercialized, is also of interest as it possesses better mechanical properties. Spider silk produced by various species of spiders vary in their amino acid content.[51] Silk has been conventionally used as a biomaterial for sutures and recently been investigated for applications in bone tissue engineering.[52]

Silk from *B. mori* is composed of two types of proteins, sercin and fibroin. Sercin forms a coating on the inner core protein, allowing self-adhesion between silk fibers; fibroin forms the core of the fiber. Fibroin, composed of nonpolar amino acids such as glycine, alanine, and serine, is made up of heavy (325 kDa) and light chains (25 kDa).[53,54] β-Pleated sheets on heavy chains form crystals in an amorphous matrix.[53,55] Silk from *Nephila clavipes*, one of the most comprehensively studied

spider silk, is composed of a single chain of fibroin (275 kDa).[51] Black braided silk in which the protein sercin has been stripped from the fiber is also used as a biomaterial for nonallergic sutures.[52]

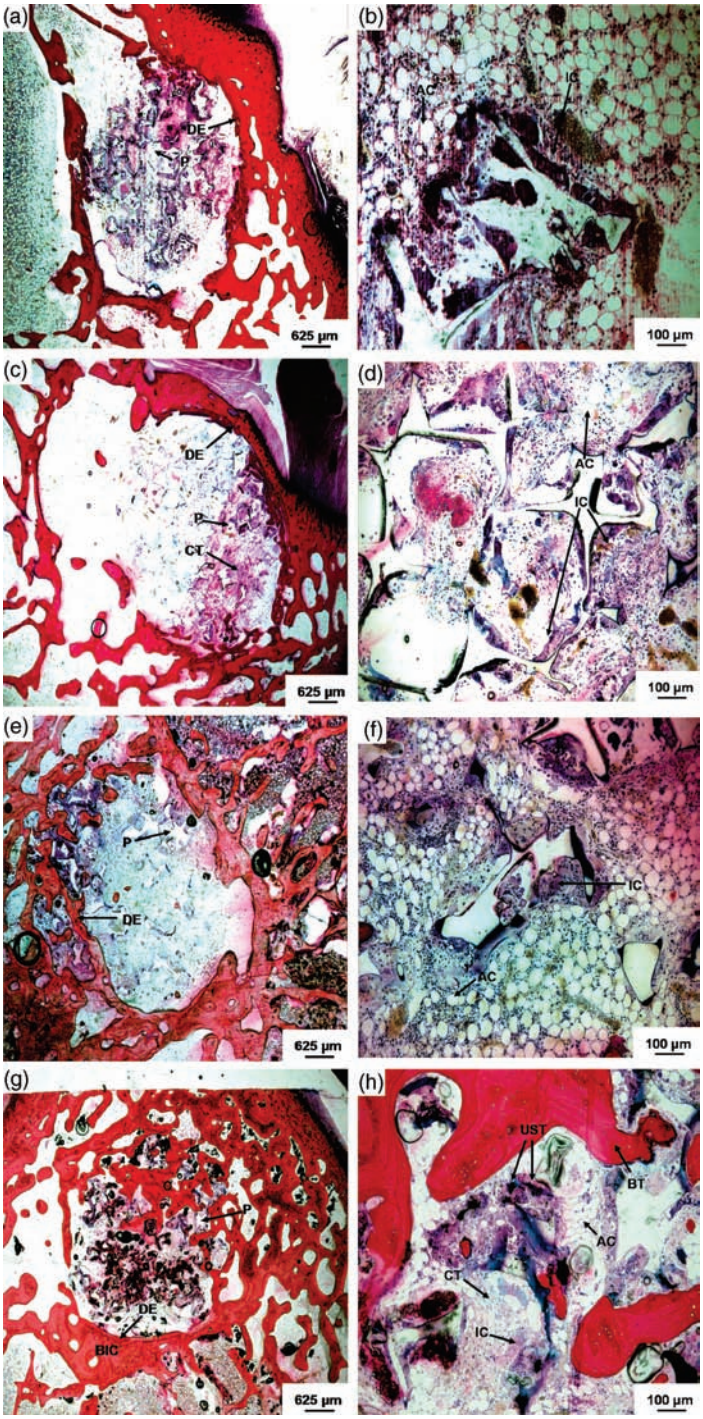
Silk line of the spider *N. clavipes* is the strongest natural fiber known.[56] Silk possesses high tensile strength and elongation capabilities. The mechanical properties of silk shows negligible changes in response to variations in strain rate. This is attributed to the decrease in viscous and elastic behavior, and an increase in the plasticity of silk fibers. However, viscosity of silk is directly proportional to strain rate, resisting elastic and plastic behavior.[57] The viscoelastic behavior of silk is a result of the stretch of amorphous regions along with the elastic deformation of β-pleated sheet crystals under stress.[55] Silk scaffolds lack sufficient mechanical properties and cannot be used to provide mechanical support to the bone structure. Silk particles have been incorporated to reinforce polymeric scaffolds, thereby increasing the mechanical properties. Compressive modulus of silk particle reinforced scaffolds is significantly silk controls.[58]

Silk scaffolds can be fabricated using electrospinning which produces nanoscale diameter silk fibers by using the same method as described in the HAp section earlier. Additionally silk scaffolds in the form of films can be developed[46] by dissolving fibrin protein in LiBr, dialyzing in water before freeze drying, and redissolving in hexafluoro-2-propanol.[14] Another method to incorporate silk into a scaffold is by creating hydrogels. To formulate hydrogels containing silk, a silk solution (created similarly to the film method by dissolving in LiBr and dialyzing in water) is mixed with ethanol in various ratios (silk solution/ethanol: 1/9, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2, and 9/1).[59]

Virgin silk fibers, originally used as suture materials, can induce hypersensitive responses in patients, characterized by an increase in IgE levels, severe allergic response, and asthma.[52] Allergic responses are attributable to the protein sercin coated on the silk fibers.[60] To reduce these adverse effects, sercin is stripped from the fibroin, creating black braided silk fibers.[52] Silk, manufactured in a twisted and braided type, uses virgin silk and is not used as a suture material. Black braided silk, which does not induce allergic responses, can lead to hypersensitivity after multiple exposures. Although not considered an allergen, black braided silk is capable of inducing a foreign body response stimulating eosinophils, macrophages, and giant cells to attack foreign material, which may become chronic due to the formation of granular tissue around the suture.[60]

Cytotoxicity of silk films, assessed by MTT assay on bone marrow stromal cells, showed that the number of cells increased significantly after 14 days.[14] Correspondingly, the cytotoxicity of silk hydrogels tested by MTS assay showed increased

FIGURE 10.4 Histologic sections of PPF scaffolds implanted in femoral condyle defects. (a and b) PPF scaffold after 4 weeks of implantation. (c and d) a US tube–PPF scaffold after 4 weeks, (e and f) a PPF scaffold after 12 weeks of implantation, (g and h) a US tube–PF scaffold after 12 weeks of implantation. The PPF scaffold appears as white areas in the image, and bonelike tissue (BT) appears red. US tubes (USTs), connective tissue (CT), adipose cells (ACs), and inflammatory cells (ICs) are also shown. Adapted from Ref. [49] © Elsevier 2008.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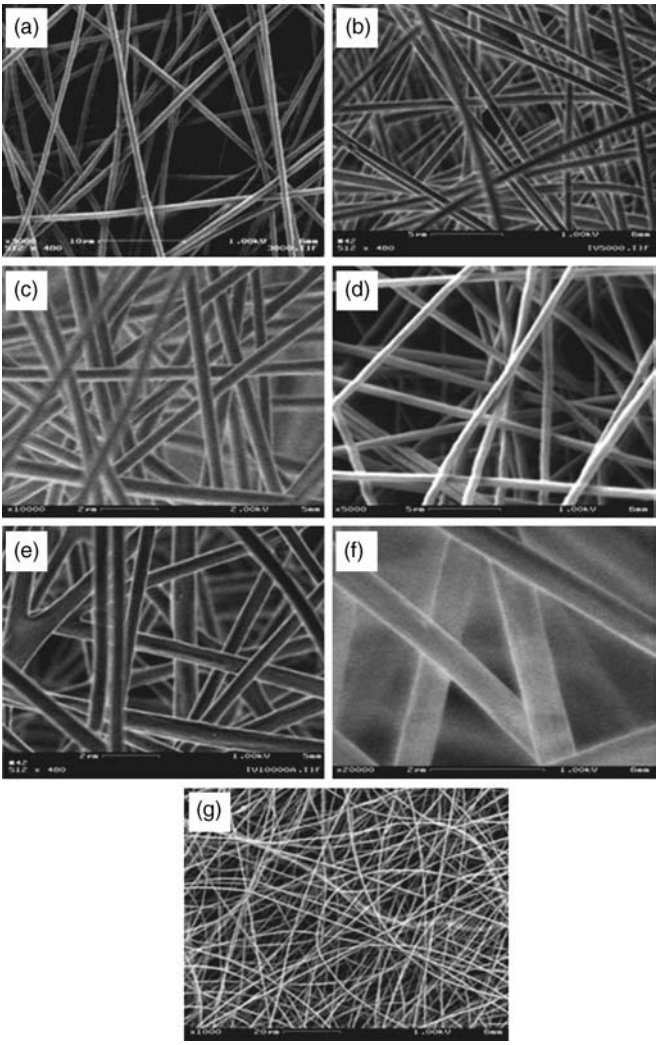

FIGURE 10.5 Scanning electron microscope micrographs of electrospun silk fibers with different diameters. (a) Fiber diameter = 840 ± 80 nm, (b) fiber diameter = 740 ± 150 nm, (c) fiber diameter = 700 ± 100 nm, (d) fiber diameter = 730 ± 50 nm, (e) fiber diameter = 720 ± 100 nm, (f) fiber diameter = 850 ± 60 nm, and (g) fiber diameter = 880 ± 50 nm. Adapted from Ref. [54] © ACS 2002.

viability of human MSCs with increasing concentration of silk after 48 h of exposure. This increase in viability is believed to be caused by an increase in β-pleated sheet crystals, elastic modulus, network size, and bound water.[59]

In nature, silk lacks cell-binding domains; however, these domains can be added to the fiber. The addition of domains renders silk fibers susceptible to macrophages,

which degrade the silk over a period of time. The addition of cell-binding domains allows native tissue growth in the matrix, allowing tissue to attain normal physiological function.[53] Addition of the peptide arginine-glycine-aspartic acid (RGD) to the surface of silk films increases cell density. In a study, films with RGD peptide on the surface had higher cell counts after 24 h, and cells continued to increase after 14 days (Fig. 10.5).[14]

In vitro studies on electrospun silk scaffolds reported cell growth and ECM formation after 14 days of incubation. Various combinations of silk, polyethylene oxide (PEO), bone morphogenic protein 2 (BMP-2), and nHAp, were used to fabricate scaffolds. BMP-2 and nHAp integrated scaffolds exhibited significant increase in calcium deposition and BMP-2 transcription levels.[35] Enhanced attachment and spreading of human MSCs and anterior cruciate ligament (ACL) fibroblasts was observed on RGD-modified silk scaffolds.[14] RGD functionalization also increases cellular mineralization; osteoblast-like cells (Saos-2) mineralized significantly on substrates containing parathyroid hormone.[55]

10.5 SUMMARY

The research to date suggests that nano- and microparticles or fibers have immense potential for applications in bone tissue engineering. Nanoparticles and nanofibers have shown to improve the mechanical properties of biodegradable polymeric implants. A few studies show that nano- and microparticle incorporated composite and scaffold implants are cytocompatible (*in vitro*) and biocompatible (*in vivo*). Some of the nanoparticles such as carbon nanotubes can also be functionalized for targeting, drug delivery, and bioimaging. Furthermore, their intrinsic physical properties can be harnessed for therapeutic and imaging applications. Although these nano- and microparticles improve the mechanical properties of bone tissue engineering scaffolds, little is known about their long-term biocompatibility and biodistribution upon their release from the scaffolds *in vivo*.[61,62] Although silk scaffolds produced by electrospinning are biocompatible, their mechanical properties can be improved by the dispersion of micro- and nanoparticles as reinforcing agents. Furthermore cell-binding domains can be modified to limit their susceptibility toward macrophage degradation. The future direction of tissue engineering field will see attempts to overcome these challenges and continue to create more biomimetic scaffolds because these nano- and microtechnologies show great promise with multifunctional capabilities for bone tissue engineering.

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11

MICRO- AND NANOTECHNOLOGY FOR VASCULAR TISSUE ENGINEERING

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11.1 INTRODUCTION

Vascular tissue engineering aims to regenerate functional blood vessels through the use of biomaterial scaffolds either preseeded with cells or designed to recruit host cells for remodeling. The field can be divided according to two overall objectives: to develop small-diameter vascular grafts with long-term patency and to generate microvascular capillary networks within large tissue-engineered scaffolds.[1,2] Both of these distinct goals benefit from recent applications of micro- and nanotechnologic innovations. Early attempts in vascular tissue engineering, especially in regard to vascular grafts, have not met expectations in part because little was known about how micro- and nanofeatures could guide cellular regeneration. As it became apparent that cells interact with their environment at multiple length scales, engineers looked to fabricate scaffolds with micro- and nanoscale architectures. This chapter discusses recent micro- and nanotechnologic approaches to tissue-engineered vascular graft design and biomaterial-driven microvascular formation.

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11.2 CONVENTIONAL VASCULAR GRAFTS

Synthetic vascular grafts have been successfully used as replacements for large vessels for several decades.[3] These grafts are typically composed of either expanded polytetrafluroethylene (ePTFE) or Dacron.[4] Despite their success as large vessel replacements, several complications arise when they are used to replace vessels with a diameter less than 6 mm. The primary reasons for failure are anastomotic intimal hyperplasia and thrombogenicity of the graft surface.[5] Intimal hyperplasia may be caused by a disruption in flow at the interface of the graft and native vessel, leading to a buildup of neotissue at the anastomosis site. Over time, this process can occlude the graft. Thrombosis on the graft surface can occur from fibrin deposition that begins immediately after implantation because of the lack of an endothelial lining. Ideally, a vascular graft would be fully covered by a confluent endothelial layer upon implantation, but synthetic grafts have shown less than 10% coverage even after several months.[6] Tissue ingrowth across the anastomotic site extends only 1–2 cm in humans and is insufficient to adequately cover a graft.[7] These two mechanisms account for nearly all the long-term failures in synthetic vascular grafts.[5] Despite much work toward overcoming these challenges, clinical results remain largely unchanged over the past few decades.

11.3 TISSUE-ENGINEERED VASCULAR GRAFTS

At a minimum, a clinically successful tissue-engineered vascular graft must meet two requirements: a confluent endothelium and a surrounding matrix for mechanical support.[1] The endothelium is essential to long-term patency to avoid platelet adhesion, coagulation, and stenosis. In native vessels, endothelial cells (ECs) form a confluent, interconnected monolayer generally aligned with the direction of blood flow. The endothelial layer is supported by the vascular basement membrane, which provides an anchorage site for ECs and can influence cellular functions through signaling pathways.[8] The basement membrane is made of extracellular matrix (ECM) protein nanofibers in a matrix of polysaccharides.[9] Tissue-engineered vascular grafts must also have sufficient mechanical properties to withstand physiological conditions and ideally would have a similar composition to native vessels. The tunica media found in smaller arteries is composed of collagen and elastin fibers with concentric layers of aligned smooth muscle cells (SMCs).[10,11] Aligned SMCs within these layers are at angles with the neighboring layers in a herringbone structure.[12] Researchers have tried to mimic both the structure and composition of native arteries to develop the ideal vascular graft.

Weinberg and Bell presented one of the first attempts at a tissue-engineered vascular graft by mimicking each layer of the native artery. The group formed SMC-laden collagen tubes supported by Dacron and seeded ECs on the luminal surface and fibroblasts on the outer surface to mimic the adventitia.[13] Although the composition was similar to physiological vessels, the resulting construct was mechanically weak. Later, Niklason et al. fabricated a SMC-seeded scaffold from biodegradable

materials and conditioned it for 8 weeks on a pulsatile bioreactor.[14,15] Afterward, ECs were attached to the lumen, and the grafts were implanted into pigs. This approach led to grafts that could withstand physiological pressures and remain patent for up to 1 month. However, when this strategy was used with human cells, the grafts were much weaker and not able to withstand physiological flow. In the past decade, much work has been done to advance the field. Other biodegradable materials, such as poly(lactic acid) (PLA), polycaprolactone (PCL), and polyhydroxybutyrate (PHB) and copolymers of these, have been examined and optimized for vascular graft applications.[16–18] ECM proteins besides collagen have been formed into vascular grafts, and methods to improve the mechanical properties through cross-linking or mechanical stimulation have been developed.[19] Material surfaces have been engineered to promote cell adhesion, display or release soluble factors, enhance diffusion, and promote cell infiltration.[20–22] Micro- and nanotechnologies have become increasingly pivotal because of the ability to interact on a cellular or macromolecular size scale. Strategies to alter cell behavior using micro- and nanotopographic cues and vascular grafts fabricated from micro- or nanofibrous materials have provided hope for a clinically successful graft.

11.4 MICRO- AND NANOTOPOGRAPHY IN VASCULAR TISSUE ENGINEERING

11.4.1 Micro- and Nanotopographies to Mimic Native Architecture

The basement membrane is a complex network of nanofibers; thus, it is not surprising that ECs have been shown to behave differently when cultured on materials with nanotopographies.[23–25] Polymer demixing techniques were used to create 13-, 35-, or 95-nm-tall islands on polystyrene-based materials in a study by Dalby et al.[26] ECs exhibited an elongated, arcuate morphology on nanotopographic surfaces compared with a flat and round shape on smooth polystyrene. The effect on cell shape was most prominent on the 13-nm islands. Chung et al. created nanoscale roughness on polyurethane (PU) films by conjugating arginine-glycine-aspartic acid (RGD)–functionalized poly(ethylene glycol) (PEG) molecules with either uniform chain length or a mixed chain length to the surface.[27] Human umbilical vein endothelial cells (HUVECs) cultured on the nano-rough films adhered and proliferated faster than on smooth surfaces. Bettinger et al. found that endothelial progenitor cell (EPC) morphology was altered by nanogrates of 600 or 1200 nm period (Fig. 11.1).[28] Whereas EPCs were more aligned and elongated, migration was enhanced compared with smooth surfaces. However, this study did not find any significant changes in endothelial gene expression, indicating that more can be done to fully promote functional endothelial layers for clinical applications.

It is likely necessary to incorporate nano- to microscale topographies into the interior surface of tissue-engineered vascular graft design. Nanofiber meshes can support confluent, interconnected EC monolayers formed from either HUVECs or outgrowth ECs from EPCs.[29] Furthermore, these monolayers showed signs of

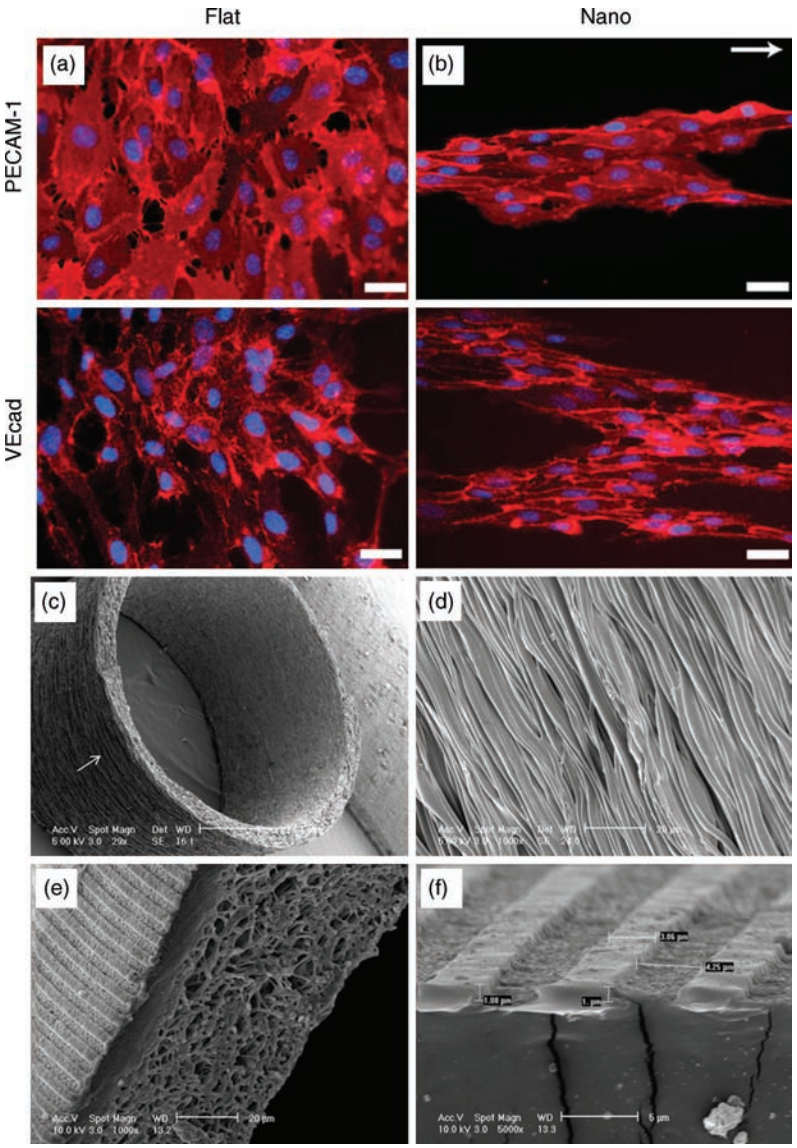

FIGURE 11.1 Endothelial progenitor cells (EPCs) cultured for 6 days on flat substrates (a). The cells form confluent layers with random orientation. EPCs cultured on nanotopography for 6 days align into multicellular structures in the direction of the topography (*arrow*) (b). The cells were stained for PECAM-1 and vascular endothelial cadherin (VEcad); scale bars are 50 μm. Adapted with permission from Ref. [28]. Scanning electron microscope (SEM) images of an electrospun PU graft with circumferentially aligned fibers (c) that are tightly packed (d). SEM images of a hybrid graft with microgrooves on the lumen surface and microfibers on the exterior (e and f). Adapted with permission from Ref. [23].

polarization and enhanced expression of integrin β1, similar to cells on physiological basement membranes. Electrospinning has been extensively used to create nanofibrous matrices for vascular grafts, and examples are discussed later in this chapter. Readers should use caution when comparing studies of EC behavior on various topographies. Liliensiek et al. studied the effects of nanoscale geometries on various types of human ECs: HUVECs, dermal microvascular ECs, aortic ECs, and saphenous vein ECs.[30] Although all cell types did respond to the rough topography, differences were seen in proliferation and migration, primarily between microvascular cells and those derived from larger vessels.

11.4.2 Microengineered Cell Sheets

Generation of a multilayered tunica media to support the vascular construct is necessary, and ideally the layers would be arranged in a herringbone fashion as seen in native vessels. Cell sheet technology is one strategy with the potential for success. L'Heureux et al. presented the first report of a vascular graft made entirely of rolled cell sheets.[31] SMCs and fibroblasts were cultured for 30 days to develop a confluent sheet that could be removed and wrapped around a mandrel. ECs were seeded onto the surface of the construct. Although only 50% patency was demonstrated after 1 week in dogs, the grafts had burst strengths similar to physiological values (∼2200 mm Hg). Further refinement of this technique led to a graft in clinical trials for arteriovenous fistula.[32]

Researchers have attempted to build upon this success by using microfabrication techniques in the production of cell sheets. Conventional cells grown on sheets are randomly oriented on the planar surface. Wong and coworkers sought to adapt their work with aligned SMCs on microgrooved surfaces to transferable cell sheets.[33,34] Williams et al. developed a technique to align SMCs on micropatterned thermoresponsive poly(*N*-isopropylacrylamide (PIPAAm) using microcontact printing.[35] Importantly, transfer of the cell sheets from the PIPAAm films did not alter the cellular morphology. A recent study demonstrated the ability to remove and stack these cell sheets in a herringbone pattern resembling native architecture using a technique called gelatin stamping.[36] It should be noted that the adhesion strength between sheets was not tested and should be optimized to function as a supporting structure for a graft. If delamination can be overcome, one could easily imagine a construct consisting of an endothelial sheet[37] surrounded by multiple, aligned SMC sheets, all rolled around a mandrel to form a cellularized vascular graft.

11.4.3 Conclusion

It is clear that topographical cues influence vascular cell behavior to a great extent. A wealth of knowledge about cellular interactions with material interfaces has been gained and is currently being applied to vascular graft design. There is much hope that nanoscale topography will allow endothelialization of graft lumens and full infiltration and remodeling of the medial layer. One area in need of investigation is

translating the fabrication techniques to vascular graft materials and geometries. These techniques must be adaptable to biodegradable or natural polymers in addition to the ability to be scaled up for manufacturing.

11.5 MICRO- AND NANOFIBROUS SCAFFOLDS IN VASCULAR TISSUE ENGINEERING

11.5.1 Nanofibrous Scaffolds

The ECM is primarily composed of nanofibers on the order of 10–500 nm in diameter and up to several micrometers in length. The nanofibers are made of several types of proteins covered in cell adhesive ligands that can regulate cell behavior. The fibers are interwoven, providing a porous mesh for cells to inhabit. The ECM plays a crucial role in differentiation, morphogenesis, and many other cellular phenomena.[38] Ideally, tissue engineering scaffolds would recapitulate the ECM to promote proper tissue regeneration. Although much research has been done on electrospinning, thermally induced phase separation and molecular self-assembly are two other methods used to produce nanofibers. However, phase separation can only be used for polymers with crystalline structure, significantly limiting its applicability in tissue engineering.[39] Also, self-assembly techniques typically do not produce materials with adequate mechanical properties or allow for control of fiber alignment.[40] Thus, the discussion will focus on recent advances in creating tissue-engineered vascular graft using electrospun fibers.

11.5.2 Electrospun Fibers

Electrospinning is an attractive approach to produce nanofibrous grafts because of its simplicity, low cost, and potential for scale-up. Fibers on the order of 50 nm to several micrometers in diameter can be formed in either random meshes or aligned using a rotating collection mandrel. The properties of the fibers such as tensile strength and diameter can be tailored by adjusting several spinning parameters such s polymer viscosity, collection plate size, geometry, and rotational speed.[41] Electrospinning has been adapted to many types of synthetic polymers, including PCL, PLA, poly(glycolic acid) (PGA), poly(lactide-*co*-glycolide) (PLGA), and polydioxane (PDO).[41–45] Vascular grafts can be created from these nanofibrous meshes (Fig. 11.1c and d) and have, in some instances, improved preclinical outcomes compared with conventional synthetic graft materials. For example, slowly degrading electrospun PCL fibers were optimized on the basis of tensile strength, fiber size, and graft morphology.[46] These grafts were tested in rats against conventional grafts made from ePTFE for 24 weeks.[47] No stenosis was seen in the electrospun PCL grafts; however, two conventional grafts were stenotic as early as 18 weeks. Both graft types demonstrated comparable neointimal formation; however, electrospun PCL grafts had significantly better endothelial coverage, immune response, and neovascularization.

Natural polymers have also been electrospun into nanofibers, including collagens types I–IV, gelatin, elastin, fibrinogen, hemoglobin, and myoglobin.[48,41,49–52] Most electrospinning approaches for natural polymers are animal derived and thus carry the limitation of immune response in the clinical arena. Human proteins have been isolated and electrospun, but the limited supply is an issue for scale up. McKenna et al. sought to develop an electrospun graft from recombinant human tropoelastin to mitigate the possibility of adverse immune reactions and supply issues.[50] ECs were able to adhere and remain viable on the scaffolds. The mechanical properties were not different from extracted elastin, providing an attractive alternative for clinical applications. However, the authors state that the mechanical properties of the elastin scaffold alone are not sufficient for implantation in a graft model.

11.5.3 Synthetic and Natural Hybrid Nanofibers

Recently, researchers have begun to create hybrid nanofibrous grafts made from a combination of synthetic and natural materials. Synthetic nanofibers generally have superior mechanical properties and are more tailorable than natural polymers. However, synthetic polymers lack key biorecognition moieties that may be imperative to promote formation of a confluent endothelium and achieve a nonthrombogenic surface. The hybrid approach is taken to yield a material with optimal characteristics. Kwon and Matsuda developed a method to co-spin poly(L-lactide-*co*-caprolactone) (PLCL) with type I collagen by mixing the two components together before spraying.[53] They assessed the properties of the grafts with a range of collagen mass fractions (0–100%). It was demonstrated that as the fraction of collagen within the mix increased, the mechanical properties of the scaffolds decreased. This result is expected given the generally superior mechanical properties of synthetic polymers. Additionally, fiber diameter was inversely proportional to collagen content, decreasing from 520 nm in 100% PLCL scaffolds to 120 nm in 100% collagen scaffolds. Cellular studies demonstrated that HUVECs could attach, become well spread, and elongate in the direction of fibers in scaffolds with 5% or 10% collagen. In a similar approach, He et al. fabricated PLA-*co*-PCL:collagen blended hybrid nanofibers with diameters in the range of 100–200 nm.[54] They found that collagen blending enhances the viability, attachment, and spreading of human coronary artery ECs. Gene expression profiles of the EC markers endothelial leukocyte adhesion molecule 1, platelet EC adhesion marker-1 (CD31), intercellular adhesion marker 1, and vascular cell adhesion marker 1 in addition to von Willebrand factor (vWF) were not significantly different from controls, indicating maintenance of the EC phenotype. Using similar grafts in a later study, the group sought to evaluate the *in vivo* performance of an acellular graft in a rabbit superficial epigastric vein model.[55] After 7 weeks, the grafts showed no macroscopic deformation. The inflammatory response was mild, with a fibrous encapsulation around the graft but no host cell infiltration. No coagulation on the lumen surface was seen; however, there were no ECs present, either. The authors stated that coagulation could have occurred at a later time point because no endothelium had developed but stressed that

implantation of cell-seeded grafts would provide a better opportunity for formation of a confluent endothelial layer. Lee et al. have developed a PCL–collagen nanofibrous scaffold with superior burst strength than native vessels or PCL fibers alone.[56] The combination of the two materials gives a higher yield strength for the composite than for a pure PCL material, which translates to a higher burst strength. When seeded with ECs and SMCs, the ECs localized to the inner luminal surface, forming a confluent monolayer, but the SMCs infiltrated the periphery of the graft. This result is encouraging because previously it had been a challenge to develop sufficiently strong nanofiber grafts with high porosity for cell infiltration.

Elastin is a major contributor to the mechanical properties of native vessels.[57] Several groups have attempted to fabricate elastin nanofiber graft or incorporate elastin within a hybrid blend.[58–60] Although pure elastin grafts lack sufficient mechanical strength to be used clinically, recent developments with blended materials hold promise. A bilayer scaffold was created by sequentially spinning elastin and PCL around a rotating mandrel.[61] This approach yielded reduced platelet adhesion and decreased thrombogenicity, which was measured by plasma clotting time. After implantation as a rabbit carotid interposition graft for 1 month, the bilayer grafts showed no reduction in size, burst strength, or compliance. Han et al. created co-spun fibers from three components, PLGA, gelatin, and elastin.[62] By altering the ratios of each component, the researchers were able to tailor key features such as fiber size, swelling characteristics, and mechanical properties. A 3:2:1 volume ratio of PLGA:gelatin:elastin yielded the smallest diameter fibers (317 nm) with the highest mechanical strength. All the variants tested were able to support cellular attachment and spreading. ECs were able to form a functional nonthrombogenic monolayer as assessed by gene expression and clotting assays.

11.5.4 Release from Nanofibers

Electrospun nanofibers are able to mimic native ECM fibers in terms of size and composition; however to fully recapitulate the cellular microenvironment, soluble factors should also be presented. Chew et al. first demonstrated the feasibility of encapsulating growth factors into electrospun fibers.[63] Human β-nerve growth factor (NGF) in a carrier protein, bovine serum albumin (BSA), was electrospun in a copolymer of PCL and poly(ethyl ethylene phosphate) (PCLEEP). Bioactive NGF was released over a period of 3 months via diffusion from the fibers. In a subsequent application to vascular grafts, heparin was incorporated in PCL nanofibers and released over a period of 14 days in a bioactive form. One limitation of incorporating the soluble factor directly into the fiber is the initial burst release. Wei et al. incorporated platelet-derived growth factor BB (PDGF-BB)–loaded microspheres into a PLLA electrospun scaffold to achieve sustained growth factor release up to 60 days.[64] The release kinetics were dependent on microsphere degradation and therefore inherently independent of the properties of the scaffold.

A more recent approach to loading nanofibers with soluble factors uses coaxial spinning technology, wherein a core material is spun inside a shell of a separate

material using two outlets. Zhang et al. demonstrated the feasibility of this approach to encapsulate proteins for release from the fibers.[65] Fluorescein isothiocyanate–BSA (FITC–BSA) was incorporated in water-soluble PEG in the core, and PCL was used as the shell material. The release properties were dependent on fiber size, with smaller fibers releasing faster because of increased ratios of surface area to volume. The study demonstrated continuous release for up to 5 months. Liao et al. extended this approach to release PDGF-BB from a BSA-core/PCL-shell nanofiber.[66] Lu et al. designed a PCL-core/cationized gelatin-shell fiber. FITC-heparin was adsorbed onto the gelatin layer, providing a platform to deliver heparin-binding growth factors within vascular grafts.[67] The group demonstrated this capability by releasing vascular endothelial growth factor (VEGF) over 15 days in a controlled manner. Future work in this area should include the release of multiple growth factors[68] with independently controlled rates and *in vivo* studies to determine the functional impact of growth factor incorporation.

11.5.5 Antithrombogenic Nanofibers

A clinically successful graft requires antithrombogenic properties to be presented until a confluent endothelium can be established on the graft lumen. Researchers have recently recognized the opportunity to present antithrombogenic signals on nanofibers to prevent platelet adhesion and coagulation within small diameter vascular grafts. Poly(ether urethane urea) (PEUU) has been electrospun with a bio-inspired phospholipid polymer, poly(2-methacryloyloxyethyl phosphorylcholine-*co*-methacryloyloxyethyl butylurethane (PMBU), to reduce its thrombogenicity.[69] These fibers were formed in 1.3 mm grafts and implanted in a rat aortic interposition model and found to increase patency and reduce thrombogenicity compared with PEUU controls. EC attachment was still allowed and a confluent monolayer formed within the 8-week time course of the study. Soletti et al. immobilized a similar phospholipid polymer (PMA, 70% 2-methacryloyloxyethyl phosphorylcholine: 30% methacrylic acid) to PEUU after electrospinning as an antithrombogenic surface functionalization.[70] The grafts were again studied as aortic replacements in rats and reduced platelet adhesion by 10-fold compared with untreated PEUU controls. Significantly more PMA-PEUU scaffolds were patent after 8 weeks (92%) compared with PEUU alone (40%).[71] Histologic evaluation of the scaffolds revealed neotissue formation with aligned collagen and elastin, confluent ECs aligned with blood flow, and SMCs in the interior of the scaffold. Recently, Liu et al. sulfated silk fibroin to mimic the highly sulfated heparin molecules found on native endothelium.[72] This biomimetic strategy reduced platelet adhesion and thrombogenicity compared with nonsulfated silk fibroin scaffolds. ECs and SMCs were cultured in the scaffolds and found to organize into confluent luminal monolayers and multilayered structures, respectively. It is critical to the success of an implanted graft to maintain an antithrombotic lumen until ECs adhere and form a confluent monolayer. These strategies are promising to reduce thrombus formation; however, it is also important to quickly recruit ECs.

11.5.6 Cell-Adhesive Nanofibers

Nanofibers can be modified by adding cell adhesive ligands to the surface to promote attachment and spreading. Kim and Park fabricated nanofibers from PLGA and PLGA-*b*-PEG-NH_2 to form amino groups on the fiber surface, to which GRGDY peptide sequences were conjugated.[73] NIH 3T3 fibroblasts were able to adhere, spread, and proliferate better on the RGD-modified fibers. A similar approach was taken by Grafahrend et al. using PEG-*b*-PDLLA electrospun fibers.[74] When RGD sequences were covalently linked, 100% of seeded cells survived after 24 h, but no living cells were found in the unmodified controls. RGD immobilized to nanofiber surfaces has also been shown in PU,[75] PCL, and P(LLA-CL)[76] with similar increases in cell adhesion, spreading, and proliferation. Other biomolecules have been conjugated to electrospun scaffolds, including type I and IV collagen, marine collagen, and chitosan of various molecular weights.[77]

11.5.7 Future Work and Conclusion

Results with electrospun nanofibrous vascular grafts offer much promise of a clinically relevant solution to small-diameter graft problems. The ability to mimic the native ECM topography, tailor the mechanical properties, release signaling molecules, and reduce thrombogenicity through antithrombogenic and EC adhesive surfaces gives them potential for future research. The field is not without limitations, however. Current methods of producing electrospun fibers have a lower size limit of around 50 nm,[9] and scaffolds are made from fibers of a few hundred nanometers in diameter.[41] This is at the upper limit of native ECM fibers, and it may be necessary to more accurately mimic the native components with smaller fibers. The fabrication process is lauded for its simplicity and potential for scale up, but it requires conditions that are not favorable for many biological entities. Harsh organics, high voltages, and processing steps can denature natural proteins. To this end, researchers have developed a method to electrospin collagen with ethanol and PBS to avoid denaturation and unwanted changes in structure.[78] Finally, the low porosity of most electrospun scaffolds limits the cellular infiltration needed for development of a truly regenerated medial layer. Numerous studies have investigated methods to increase the scaffold porosity, but most techniques significantly reduce the mechanical properties of the scaffold. Recently, groups have shown that cells can be electrosprayed in the core of a coaxial electrospinning cone to form fibers with encapsulated cells.[79–81] This approach, which was shown to maintain cellular viability, negates the need for cellular infiltration because the cells can be uniformly distributed throughout the construct. However, cell-mediated remodeling of the graft structure is also dependent on porosity. Therefore, simply seeding cells throughout a construct may not be sufficient to establish a functional medial layer. Others have developed bi- and trilayered scaffold to optimize the properties of each layer toward specific goals (Fig. 11.1e and f). Ju et al. developed a scaffold with an inner layer made from 270-nm PCL fibers surrounded by a highly porous outer layer of micron-

sized fibers.[82] ECs attached to the inner nanoscale fibers in a confluent monolayer, which was confirmed by CD31 staining. SMCs were shown to infiltrate the more porous outer layer, with α-smooth muscle actin (SMA) present throughout.

Electrospinning nanofibers is a simple and controllable method to produce ECM-mimicking fibers to fabricate tissue-engineered vascular grafts. Engineers must continue to adapt electrospinning techniques to overcome the current limitations. Additionally, progress had been made by combining electrospun fibers with other micro- and nanofabrication techniques. Researchers should continue to look for synergistic combinations to produce clinically successful tissue-engineered vascular grafts.

11.6 MICROVASCULAR TISSUE ENGINEERING

11.6.1 Need for Microvascular Networks in Tissue Engineering

Apart from designing macroscale vascular constructs, vascular tissue engineering is focused on *de novo* development of microvascular networks. Thin or avascular tissue engineering products have been successful in the clinic,[83–85] but bulk constructs have proven challenging because cells seeded within the scaffolds must rely on diffusion to provide oxygen and nutrients necessary for viability, proliferation, and remodeling. Thus, the thickness of the construct becomes the limiting factor. Studies have shown that cells cannot survive more than a few hundred micrometers from a capillary source. [2,86] Creating or developing robust vascular networks within tissue engineering scaffolds is critical to the continued success of the field. Toward this goal, microfluidics and microfabrication techniques have been used to gain precise control of geometry, architecture, and flow within a construct.

11.6.2 Microfluidics

As awareness of the need to vascularize tissue-engineered constructs became apparent, researchers looked to the field of microfluidics as an attractive system for controlling size, branching, and flow in a precise manner. The initial contributions in this field were made by Bornstein and Vacanti. Bifurcated patterns were fabricated using photolithography in silicon and Pyrex, and ECs and hepatocytes were cultured in the device (Fig. 11.2a and b).[87,88] The cells were lifted from the surface as a monolayer and maintained their proliferative capacity and functionality. The lifted ECs also aligned to form branched networks, reminiscent of native capillary structures. Further studies were performed using soft lithography to mold polydimethylsiloxane (PDMS) on silicon wafers.[89]

Although this pioneering work demonstrated the ability to engineer microfluidic systems and successfully culture cells within them, the materials used did not lend themselves to tissue engineering applications because of their limited biocompatibility and nonbiodegradable nature.[90] King et al. used the biodegradable polymer PLGA to form microfluidic systems.[91] However, PLGA is a brittle material

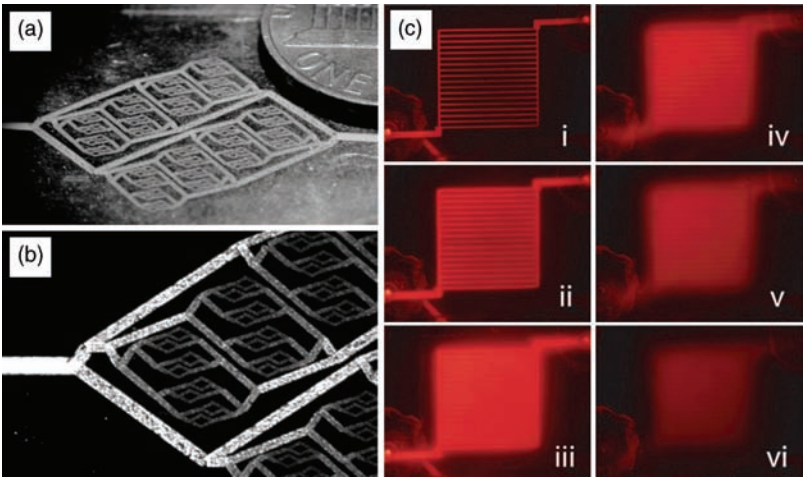

FIGURE 11.2 Multilevel microchannel network (a and b). Branch dimensions range from 130 to 660 μm. Reproduced with permission from Ref. [88]. Demonstration of spatiotemporal control of macromolecules in hydrogel networks (c). Microfluidic network formed in an alginate hydrogel showing assisted delivery (i–iii) and assisted extraction (iv–vi) of fluorescent conjugated dextran (70 kDa). Adapted with permission from Ref. [94].

that is not ideal for microvascular networks. Wang and coworkers were able to engineer microfluidic systems with elastomeric poly(glycerol sebacate) (PGS) as a more flexible alternative.[92]

Three-dimensional scaffolds are necessary to recapitulate the native environment and fully perfuse a scaffold. It still remains a challenge to develop 3D microfluidic systems, and most success has been had in stacking 2D films to create a 3D composite. Successful stacking was first demonstrated by King et al. in PLGA[91] and Bettinger et al. in PGS,[93] both using thermally bonding layers of 2D films. The PGS construct was able to exhibit maximum shear stress throughout each channel, making it attractive for microvascular systems. Unfortunately, stacking of films is not ideal because it is difficult to scale.

11.6.3 Microfluidic Hydrogels

Microfabrication techniques were recently applied to hydrogel materials to yield constructs amenable to cell culture and vascularization with precise control of architecture in three dimensions. Cabodi et al. first demonstrated the creation of a microfluidic hydrogel using soft lithographic techniques with calcium alginate, a commonly used biomaterial whose ionic cross-links can be reversed with a calcium chelator.[94] In this work, the group created slabs of alginate and bonded them together by first treating the surfaces with sodium citrate, a chelator, and then adding calcium chloride to seal the layers. This layer-by-layer approach yielded a microfluidic gel with minimum channel dimensions of 25 μm. The group also demonstrated that the gel was permeable to both small molecule and macromolecular solutes, as needed in

tissue engineering applications (Fig. 11.2c). The Stroock group later demonstrated the ability to fabricate these gels in the presence of cells and maintain their viability.[95] Capitalizing on the microfluidic channels and permeability of the gels, the group demonstrated supreme spatial and temporal control of both large and small solutes. Additionally, multiple independent networks could be used as sources or sinks to establish and sustain concentration gradients within the constructs. It has been shown that many angiogenic processes are enhanced or even dependent on the presence of concentration gradients. These seminal studies established a basis for growth, and recently microfluidic and microfabrication approaches have been extended into a variety of hydrogel and 3D tissue engineering constructs.[96–101]

11.6.4 Micropatterning

Patterning microchannels into scaffolds has been proposed as an attractive mechanism to drive vascular formation and infiltration deep within a construct. Bryant et al. created porous poly(hydroxyethylmethacrylate) (polyHEMA) scaffolds with patterned channels between 200 and 500 μm using photo-patterning techniques.[96] In this work, a photomask was used to block UV irradiation of a polymer precursor solution, which could be washed away from masked regions after irradiation. However, construct thickness is limited by the relatively shallow penetration of conventional UV patterning techniques. Other limitations of this approach include detrimental effects from exposure of cells to UV light and solvents. Another study from the Ratner laboratory showed the fabrication of a porous poly(HEMA)-*co*-(methacrylic acid) scaffold with channels by using sacrificial polycarbonate fibers embedded within the construct upon initial polymerization.[97] The fibers were dissolved afterward, leaving parallel channels of the diameter of the fiber, which allows for precise control of the diameter and spacing of the channels, with uniform properties throughout the depth of the scaffold. Scaffolds with 60 μm channels were implanted into rat myocardium for 4 weeks and found to enhance the neovascular response. By perfusing the rats before sacrifice, the group demonstrated functional vessels throughout the scaffold that successfully inosculated with the host. Additionally, SMCs were found surrounding ECs, suggesting mature vasculature had formed.

The Stroock group sought to transition to materials that could be remodeled by cells because they are more usable in tissue engineering applications. Collagen gels were selected because they would allow adhesion, proliferation, and modification by ECs. The group chose to use dense collagen gels to achieve superior mechanical properties without altering the structure or function of collagen. Micromolding was used to create patterned channels within the gels and could be successfully performed on gel concentrations as low as 0.3%.[98] Again, diffusion of large macromolecules (dextran 70 kDa) was shown. HUVECs that were seeded onto the channels showed attachment and the ability to remodel the matrix through either displacement or degradation. Tube formation was evident within 3 days, and networks grew over time. Finally, the study demonstrated that HUVECs could

invade the gels, with invasion distance and speed inversely proportional to collagen concentration. Zheng et al. recently created microstructured pores, slots, or networks within collagen and calcium alginate gels and investigated the vascularization response in a subcutaneous model in rats.[99] Circular channels of 100–400 μm diameter and 400 μm depth, microslots 100 μm wide and 400 μm deep, or a double-layered combination of the two could be fabricated in bulk gel constructs that were 1 mm thick and 8 mm in diameter. Vascularization was seen all the way into the depths of the channels or slots, with lateral invasion into the gel constructs by day 14. Even double-layered structures were vascularized throughout the gels; however, no vascularization was seen in nonstructured control gels at the same time point. CD31, SMA, and presence of blood cells indicated that the vessels were mature, stable, and inosculated with the host. The invading cell density was found to decrease with increasing structure size. This work demonstrated successful guidance of cell ingrowth and vascular formation with spatial control to 100 μm *in vivo*.

In another recent contribution, PDMS molds were used to form collagen gels within microchannels.[100] Microvascular cells could be seeded within the gels by centrifugation and were shown to form tubes between 24 and 48 h in culture with media containing basic fibroblast growth factor (bFGF) and VEGF. The group was able to control tubulogenesis dynamics and tube diameters by altering the channel geometries and collagen concentrations. Wider, elliptical shaped channels formed larger tubes, as did channels filled with higher concentrations of collagen. Tubes formed more rapidly in channels filled with lower concentrations of collagen. Branched structures were created, and the researchers demonstrated that tubes could be formed to the shape of the mold, with patent lumens throughout the branches, illustrated by staining. Finally, the study showed that the tubes could be encapsulated into a biological matrix by applying ungelled collagen over the channels and cross-linking it, resulting in a bulk structure with spatially controlled EC tubes throughout. The implications in this approach for design of bulk tissue engineering constructs are apparent. However, because of the planar nature of the design, a layer-by-layer approach would be necessary for 3D constructs, and scale up would be difficult. Gillette et al. demonstrated the formation of branched networks within bulk phase natural polymer hydrogels.[101] These channels were filled with another patterned phase hydrogel that is anchored to the bulk hydrogel through *in situ* collagen fiber assembly (Fig. 11.3). Using this technique, a variety of material combinations of collagen, fibrin, alginate, or matrigel can be used to fabricate cell-seeded branched networks within large tissue engineering matrices.

11.6.5 Hybrid or Advanced Approaches

Sundararaghavan et al. sought to combine the ECM-mimicking properties of electrospun fibers with the geometrical control afforded by photopatterning.[102] They spun methacrylated hyaluronic acid (HA) with poly(ethylene oxide) (PEO) into random or aligned nanofibrous mats. The mats were polymerized with UV irradiation and could be patterned with a photomask to either 165 or 333 μm diameter channels. Subcutaneous implants of the scaffolds revealed large vascular

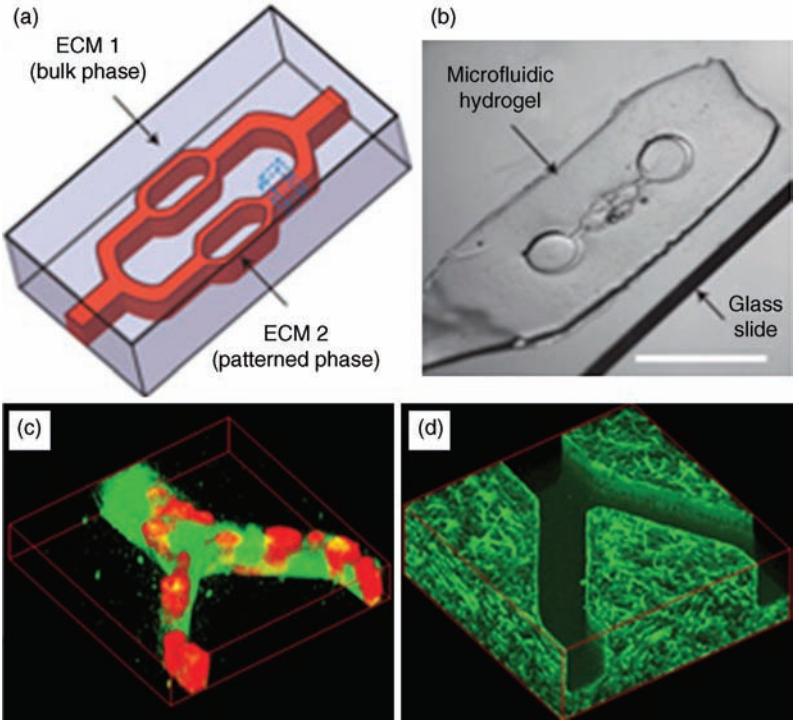

FIGURE 11.3 Schematic of microfluidic hydrogel with two phases of ECM proteins (bulk and patterned) (a). Image of corresponding microfluidic hydrogel; scale bar is 5 mm (b). Confocal reflectance microscopy of patterned collagen (*green*) seeded with HUVECs (*red*) demonstrates complete filling of the channel and formation of sharp boundaries (c). Box dimensions are 230 μm × 230 μm × 30 μm. Collagen–alginate bulk phase before patterned collagen is added, demonstrating precise geometry and uniform distribution of bulk phase fibers (d). Box dimensions are 230 μm × 230 μm × 75 μm. Adapted with permission from Ref. [101].

structures within the channels after 1 week but found no vascularization in non-patterned scaffolds.

Others have recently found unconventional methods to generate patterned structures for microvascular formation. Sadr et al. attached HUVECs to gold microrods using an oligopeptide self-assembled monolayer (SAM) that was electrochemically cleavable.[103] Methacrylated gelatin (GelMA) was poured over the rods and cross-linked with UV light. By applying an electric potential across the rods, the SAMs were cleaved resulting in efficient transfer of HUVEC monolayers to the walls of channels formed around the rods in the GelMA constructs. The group also demonstrated the ability to form a layer of NIH 3T3 fibroblast support cells around the HUVEC monolayer by dip coating the HUVEC covered rods in a solution of GelMA:3T3s. Other work in the Khademhosseini laboratory has focused on the sequential assembly of microgels to form vascular-like constructs.[104] Doughnut-shaped PEG microgels loaded or coated with HUVECs could be assembled into

tubes by sequentially detaching them from a surface in a hydrophobic medium. Because of the nature of sequential assembly, the inner diameters of the channels can be modified, and bifurcations can even be introduced, with resolution proportional to the thickness of the microgels. Concentric microgels were also formed with SMCs on the external surface and HUVECs on the inner surface.

Huang et al. created tree-like structures in poly(methyl methacrylate) (PMMA) blocks using electron beam lithography generate a charge within the material and then grounding it to induce a rapid discharge.[105] The intense discharge leads to vaporization and fracture within the block, resembling branched microvascular networks with a thick trunk around 1 mm that tapers to many endpoints on the order of 10 μm in diameter. By forming multiple nucleation sites before discharge, interconnected networks could be created with "ports" at several locations. The group also demonstrated the feasibility of this technology in biodegradable PLA blocks; however, a narrower channel range was found (20–300 μm). Cellular studies still need to be performed to assess the practicability of this technique for vascularization.

11.6.6 Nanofiber Gels

Recapitulation of the ECM is also desirable to induce microvascular formation. As previously discussed, electrospinning is a simple and convenient technique to form nanofibers. However, electrospinning is only capable of producing fibers down to approximately 50 nm. ECM fibers can be as small as 10 nm, prompting researchers to develop other techniques to reach the lower end of the spectrum. The self-assembly approach is attractive for its ability to produce fibers on the order of 10 nm and incorporate biomimetic moieties.

The Stupp laboratory has pioneered the field of self-assembling peptide amphiphiles (PAs). The group has designed the PAs around four basic functional units: (1) a hydrophobic moiety, (2) a β-sheet forming peptide sequence, (3) one to three charged amino acids, and (4) a bioactive signaling epitope that is displayed on the surface of the fiber.[106] Each of these domains can be tailored to fit a particular application. For example, the gel's mechanical properties, gelation kinetics, and nanostructure are largely dependent on the particular β-sheet unit used. The bioactive signaling unit is not necessary for fiber formation but is used to direct cells. Using this PA approach, fibers can be formed between 6 and 12 nm wide and up to several micrometers in length. The resulting nanofiber gels have a storage modulus around 10 kPa.

Narmoneva et al. developed hydrogels from ionic self-complementary peptides that underwent self-assembly.[107] Cardiomyocytes were cultured in these gels alone, with ECs, or with ECs that were cultured for 24 h before cardiomyocyte seeding to develop "prevascularized" networks. Both EC co-culture groups also promoted expression of the gap junction protein connexin 43. Preformed EC networks demonstrated a functional contribution by increasing spontaneous contractility of the cardiomyocytes by three orders of magnitude. A separate study investigated the effects of these gels for *in vivo* vascularization in a myocardial injection model in mice.[108] The nanofiber gels were found to recruit EC progenitors and vascular SMCs

that formed vascular structures. Injected neonatal cardiomyocytes were able to survive and proliferate while recruiting endogenous cells.

Physical matrix induction cues can be enhanced with the addition of soluble factors. The Stupp group sought to bind and release proangiogenic molecules within the nanofiber gels to provide physical and chemical signals for the encapsulated cells. Rajangam et al. found that the Cardin–Weintraub heparin-binding domain could be incorporated into the PAs.[109] These PAs self-assembled into nanofiber gels in the presence of heparin and demonstrated prolonged release of bound protein for more than 10 days. The gels promoted significant neovascularization in a rat corneal angiogenesis assay when loaded with small amounts of VEGF or FGF-2. A dorsal skin fold chamber and a subcutaneous implant model were used to further analyze the performance of the gels *in vivo*.[110] In both models, neovascular formation was promoted, and the inflammatory response was minimal. Furthermore, the gels were shown to persist for at least 30 days in the subcutaneous environment.

Self-assembly nanofibers provide a technique to form small ECM-mimicking fibers with tailorable mechanical properties to promote vascularization. However, a challenge for the field has been the difficulty to form complex structures with this approach. Very recently, PA chemistry was also translated to form a peptide-based membrane construct to bind and release growth factors and promote cell adhesion.[111] HA and a cationic PA were combined to form a self-assembled PA-HA hybrid membrane. By incorporating the heparin-binding domain again, the group was able to bind heparin-binding growth factors within the membrane and release them over time. Additionally, mesenchymal stem cells (MSCs) could adhere and proliferate on the membranes. In a chick allantoic membrane model, the membranes induced rapid and robust angiogenesis when loaded with small amount of growth factors compared with unloaded membranes or soluble growth factors alone. Formation of complex structures is an important step for self-assembly nanofibers, and future work to create other structures using self-assembly holds much potential.

Natural materials can also be used to form nanofibrous gels with the ability to direct cell behavior. These gels can be modified to enhance their mechanical properties, load and release growth factors, or guide differentiation. Our laboratory has developed a chemically modified (PEGylated) fibrin gel (Fig. 11.4a) with tailorable properties such as fiber diameter and storage modulus based on the type of PEG used.[112,113] Notably, the initial fiber diameter and storage modulus of unmodified fibrin can be either increased or decreased (Fig. 11.4b–d). We have demonstrated differentiation of MSCs to an endothelial phenotype and the formation of tubes *in vitro* (Fig. 11.4e–g). The cellular response is dependent on the specific PEG used and is therefore tailorable. We have also demonstrated loading and release of multiple growth factors within the gel.[68] The growth factors can be loaded via physical affinity for the fibrin matrix or by covalent conjugation to the PEG chains.

11.6.7 Conclusion

Micro- and nanotechnology have provided techniques to make great strides in the formation of microvascular networks for tissue engineering scaffolds. Microfluidics

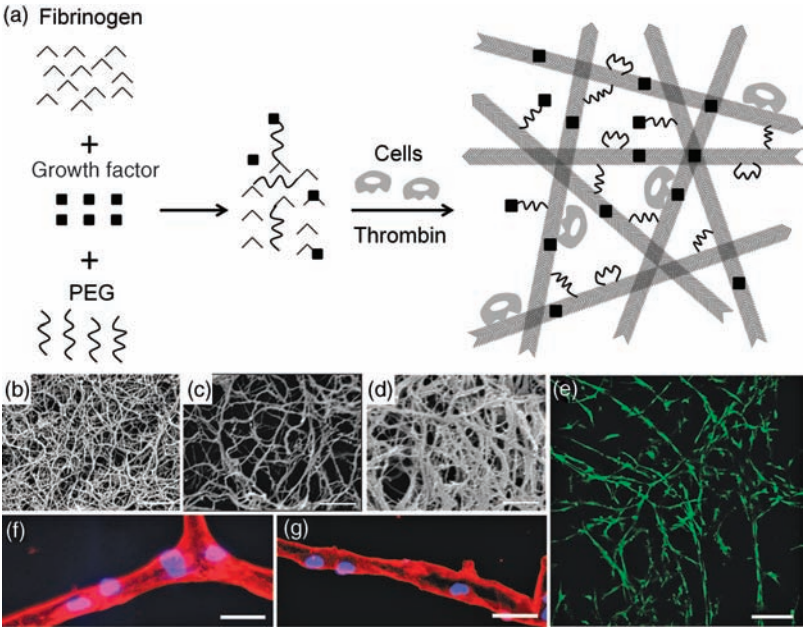

FIGURE 11.4 Schematic diagram of fibrin PEGylation, growth factor loading, and thrombin-mediated cross-linking in the presence of cells (a). Scanning electron microscope images of fibrous network demonstrating the ability to tune fiber diameter with PEG type. Fibrin-only fiber diameter was 175 nm (b), NHS-PEG was 130 nm (c), and SMB-PEG was 220 nm (d). Scale bar is 1 μm. Confocal microscopy Z-stack of calcein-AM stained human mesenchymal stem cell (hMSC) network in PEGylated fibrin gels demonstrating a robust tube network (e). Scale bar is 250 μm. Immunostaining for CD31 (f) and vWF (g) of hMSCs in PEGylated fibrin gels. Scale bar is 10 μm. Parts b, c, d, f, and g are adapted with permission from Ref. [113].

allow supreme spatial control of cell-laden channels and other conditions within the matrix. Microfabrication techniques allow researchers to direct tube formation *in vitro* or guide vascular invasion *in vivo*. Nanofiber gels have shown the ability to induce neovascularization, bind angiogenic molecules, and function as membranes for tissue engineering applications. Engineers must continue to adapt other technologies to vascular tissue engineering to achieve success.

11.7 CONCLUSIONS

Contributions to the field of vascular tissue engineering from micro- and nanotechnology are numerous. Toward the development of tissue-engineered vascular grafts, they include surface topography to direct vascular cells, micropatterned cell sheets, and nanofibrous matrices for scaffolds. Additionally, microfluidic systems to control flow and cell location have given us methods to study angiogenesis under

tightly regulated conditions. Finally, microfabricated scaffolds and nanofiber gels have been designed to enhance vascularization *in vivo*, bringing tissue engineering one step closer to clinical success.

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12

APPLICATION OF STEM CELLS IN ISCHEMIC HEART DISEASE

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12.1 INTRODUCTION

Many diseases kill cells in the organs, claiming lives or impairing a person's ability to live a normal life. For example, about 5.8 million Americans have heart failure, and 700,000 are diagnosed with it each year (Centers for Disease Control). In heart failure, much of heart muscle itself dies, so the heart cannot sufficiently pump blood.

Similarly, about 24.6 million Americans and 5% of adult Indians have diabetes. About 5–10% of these people have type I diabetes in which the insulin-producing cells of the pancreas are dead. Finally, about 1 million Americans live with Parkinson's disease. In this disease, cells that make the neurotransmitter dopamine, which helps control movement, die. Patients with Parkinson's disease have tremors and uncontrollable movements. Could these patients be treated and live normal lives? That is the goal of stem cell research.

Heart disease is an endemic health problem of great magnitude in the world. Despite considerable clinical and research effort during the past decade and the development of new drugs and surgical modalities of therapy, the mortality and morbidity remain very high. Because the limited potential of the myocardium for self-repair and renewal, a significant proportion of cardiac muscle loses its ability to perform work; this loss may be the most important factor in the heart pump failure occurring in patients with coronary artery disease and dilated cardiomyopathy. Until

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recently, reperfusion of the ischemic myocardium was the only intervention available to restore the various cellular functions affected by myocardial ischemia, including preventing cell death by necrosis or apoptosis. Unfortunately, reperfusion may result in extensive myocardial damage, including myocardial stunning, and the functional recovery of the heart may appear only after a period of cardiac contractile dysfunction that may last for several hours or days. It is evident that the limited capacity of regeneration and proliferation of human cardiomyocytes can prevent neither the scar formation that occurs after myocardial infarction (MI) nor the loss of heart function occurring in patients with cardiomyopathy and heart failure. Replacement and regeneration of functional cardiac muscle is an important goal that could be achieved either by stimulation of autologous resident cardiomyocytes or by the transplantation of allogenic cells (e.g., embryonic stem cells, bone marrow mesenchymal cells, or skeletal myoblast).[1–24]

The discovery of cardiogenesis in adult animals and human represent one of the most significant advances in cardiology in the past 25 years (Table 12.1). Previously, most cardiologists believed that the birth of new cardiomyocytes was only confined to the fetal and neonatal heart. This dogma recently collapsed when researchers discovered that the hearts of adult rats, mice, and humans undergo significant cardiac

TABLE 12.1 Major Milestones in Stem Cell Research

1981. First mouse embryonic stem cell (ESC) lines isolated and grown in culture
1981. First transgenic animals produced
1988. First cord blood transplant
1989. A clonal line of human embryonal carcinoma cells derived
1990. Britain passes the Human Fertilization and Embryology Act
1994. Human ES-like cells generated
1995. Evidence found for neural stem cells
1996. Dolly, the first cloned sheep, born in Scotland
1998. Scientists at University of Wisconsin-Madison and Johns Hopkins University isolated the first human stem cells
2000. Scientists in Singapore and Australia derive human ESCs from blastocysts
2001. Advanced Cell Technology creates the first cloned human embryos
2001. U.S. President George Bush blocks federal funding for creation of new stem Cell lines
2001. Human ESCs successfully developed into blood cells
2002. Neural stem cells successfully developed into functional neurons
2003. Institute of Stem Cell Research, Edinburgh, discovers key gene that keeps ESCs in a state of youthful immortality
2003. Dolly dies after developing progressive lung disease
2003. UK Stem Cell Bank established
2004. Web-based resource for international stem cell researchers launched
2004. Californians approve Proposition 71 to spend $3 billion over 10 years on stem cell research
2005. U.S. House of Representatives approves a bill to loosen restrictions on federal funding for stem cell research
2006. Judge rules in favor of proceeding with the financing of California's 10-year stem cell project (based on Proposition 71)

changes as a function of age. New cardiomyocytes are can be produced by, homing in to myocardial areas relevant to cardiac pathways; then they integrate structurally so that myocardial function can be restored and new tissue can be produced.[25–36]

12.1.1 Potential Uses of Human Stem Cells

New drugs have been tested for safety on differentiated cells generated from human pluripotent cell lines. Cancer cell lines, for example, are used to screen potential antitumor drugs. The generation of cells and tissues can be used for cell-based therapies; replacement cells; and tissues to treat diseases such as Parkinson's and Alzheimer's diseases, spinal cord injury, stroke, burns, heart disease, diabetes, osteoarthritis, and rheumatoid arthritis.

12.1.2 Various Sources of Stem Cells

Table 12.2 lists sources of stem cells.

Technical challenges with stem cells include the ability to obtain source material (ethical, concerns, abundance, vs. rarity), ability to direct differentiation and to select out and purify desired phenotypes, engraftment and integration versus migration, tolerance versus rejection, and tumor formation *in vivo*.

12.1.3 Unique Properties of Stem Cells

Stem cells have three general properties:

1. They are capable of dividing and renewing for longer periods.
2. They are unspecialized.
3. They can give rise to specialized cell types.

Stem cells are unspecialized. They do not have any tissue-specific structures that allow them to perform specialized functions. They cannot work with their neighbors

TABLE 12.2 Sources of Stem Cells

Adult tissue
Hematopoietic stem cells
Mesenchymal stem cells
Multipotent adult progenitor cells
Neural stem cells
Muscle-derived stem cells
Pancreatic stem cells
Hepatic stem cells
Epithelial stem cells
Cord blood and placenta
Fetal tissue
Embryos (embryonic stem cells)

to pump blood through the body (like a heart muscle cell), cannot carry molecules of oxygen through the bloodstream (like a red blood cell), and cannot fire electrochemical signals to other cells that allow the body to move or speak (like a nerve cell). However, unspecialized stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells.

12.1.4 Stem Cells Can Give Rise to Specialized Cells

Unspecialized stem cells become specialized cells; the process is called differentiation. The internal signals are controlled by a cell's genes. The external signals include chemicals secreted by other cells and physical contact with neighboring cells and certain molecules in the microenvironment.[11,37] It is unknown whether internal and external signals for cell differentiation are similar for all kinds of stem cells. It is also not known whether specific sets of signals promote differentiation into specific cell types.

Stem cells are essentially the building blocks of the human body. Stem cells are capable of dividing for long periods of time; they are unspecialized but can develop into specialized cells. The earliest stem cells in the human body are those found in embryos. The stem cells inside an embryo will eventually give rise to every cell, tissue, and organ in the fetus's body. Unlike a regular cell, which can only replicate to create more of its own kind of cell, a stem cell is pluripotent. When it divides, it can make any one of the 220 different cells in the human body. Stem cells also have the capability to self-renew; they can reproduce themselves many times over.[12] Commonly, stem cells originate from two main sources, and they are embryonic stem cells (ESCs) ESCs include those found within the embryo, the fetus or the umbilical cord blood. Depending on when they are harvested, ESCs can give rise to just about any cell in the human body.

12.1.4.1 Adult Stem Cells Adult stem cells can be found in infants, children, and adults. They reside in already developed tissues such as those of the heart, brain, and kidney. They usually give rise to cells within their resident organs. However, unspecialized stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells.

12.1.5 Embryonic Stem Cells

Embryonic stem cells are the most primitive of all stem cells. They develop as the inner cell mass in the human blastocyst derived from a 4- or 5-day-old human embryo that is in the blastocyst phase of development. The embryos are usually extras that have been created in *in vitro* fertilization (IVF) clinics, where several eggs are fertilized in a test tube but only one is implanted into a woman. Sexual reproduction begins when a male's sperm fertilizes a female's ovum (egg) to form a single cell called a zygote. The single zygote cell then begins a series of divisions, forming 2, 4, 8, 16 cells and so on. After 4–6 days before implantation in the uterus, this mass of cells is called a blastocyst. The blastocyst consists of an inner

cell mass (embryoblast) and an outer cell mass (trophoblast). The outer cell mass becomes part of the placenta, and the inner cell mass is the group of cells that will differentiate to become all the structures of an adult organism. This latter mass is the source of ESCs—totipotent cells.[4,16,22,37–40]

In a normal pregnancy, the blastocyst stage continues until implantation of the embryo in the uterus, at which point the embryo is referred to as a fetus. This usually occurs by the end of the 10th week of gestation after all major organs of the body have been created. However, when extracting ESCs, the blastocyst stage signals when to isolate stem cells by placing the "inner cell mass" of the blastocyst into a culture dish containing a nutrient-rich broth. Eventually, these undifferentiated cells can be stimulated to create specialized cells. In culture, they spontaneously form cystic structures known as embryoid bodies. Lacking the necessary stimulation to differentiate, they begin to divide and replicate while maintaining their ability to become any cell type in the human body.

The beating embryoid bodies contain a mixed population of newly differentiated cell types, including cardiomyocytes, based on the expression of cardiac-specific genes such as cardiac-myosin heavy chain; cardiac troponin I and T; atrial natriuretic factor; and cardiac transcription factors GATA-4, Nkx2.5, and MEF-2; cellular ultrastructure; and extracellular electrical activity. These cardiomyocytes can be of the pacemaker atrium- and ventricle-like type, and they are distinguishable by their specific patterns of action potential. Although the precise cellular and molecular events comprising the pathway of ESC cardiomyocytes specific differentiation remain largely undetermined, significant progress has been made in identifying the regulatory factors that can enhance or inhibit the process. Differentiation into a particular cell type depends on these factors. For instance, inhibition of bone morphogenetic protein (BMP) signaling by its antagonist Noggin induces cardiomyocytes differentiation from mouse ESCs, and retinoic acid specifically induces the formation of ventricular-specific cardiomyocytes. Nitric oxide (NO), generated either by NO synthase activity or exogenous NO exposure, has also been implicated in the promotion of cardiomyocyte-specific differentiation from mouse ESCs. Cardiomyocyte differentiation of human ESCs could be enhanced by treatment with 5-aza-2′-deoxycytidine. Also, insulin-like growth factor 1 promotes cardiomyocyte differentiation phenotype and the expression of the cardiomyocyte phenotype in ESCs *in vivo*. Interestingly, increased levels of oxidative stress appear to reduce the cardiotypic development of embryoid bodies.[12,41–43] ESC-derived cardiomyocytes show macromolecular sarcomeric organization, which gives rise to calcium sparks and ionic currents and leads to functional and anatomical integration with surrounding cardiomyocytes, which leads to propagation of electrical activity as well as pacemaker activity. ESCs spontaneously differentiate into fully functionally active, fetal-like cardiomyocytes *in vitro*. Studies have shown a dose-dependent incidence of tumor formation, particularly teratocarcinoma formation[44] (Table 12.3).

Various chemicals and molecules have been used to enhance cardiomyogenic differentiation of ESCs, including retinoic acid,[4] ascorbic acid, transforming growth factor (TGF), and BMPs.[45–52] More recently, human ESC-derived cardiomyocytes have been shown to successfully engraft and electromechanically integrate when

TABLE 12.3 Markers of Stem Cell–Derived Cardiomyocyte Differentiation

Cell Type	Differentiation Agent	Markers of Differentiated Cardiomyocyte
ES cells		
ESCs	IGF-1, TGF-β	α-Sarcomeric actin, connexin 43, MHC I, sarcomeric myosin
P19 embryonal carcinoma line	5′-Azacytidine	BMP-2, BMP-4, Bmpr la, Smadl, GATA-4, Nkx2.5, cardiac troponin I, desmin
BMC		
Bone marrow (MSCs)	Insulin, ascorbic acid, dexamethasone	α-Skeletal actin, β-MHC, MLC-2v, CaV1.2, cardiac troponin I, sarcomeric tropomyosin, cardiac titin
Cardiac stem cells		
CKIT + Lin–	NA	c-kit+
isll+	NA	Csx/Nkx-2.5, GATA4
Sca-1+ cKit–	5′-Azacytidine oxytocin	High telomerase activity, Sca-1+ Csx/Nkx-2.5, GATA4, MEF-2C, α + β–MHC, MLC-2, cardiac-α actin
Cardiosphere	cKit+	Cardiac troponin I, myosin heavy chain, atrial natriuretic peptide
SP cells	NA	ATP-binding cassette transporter (ABCG2)

NA, not available.
Source: Adapted from Ref. [17].

injected into uninjured hearts. In these studies, the injected cells behaved as a biological pacemaker and electrically excited the rest of the ventricle.[7,53] Problems are large-scale generation of ESC–derived cardiomyocytes, immunologic rejection, differentiation into undesirable lineages, and ethical concerns.

12.1.6 Recommendations

The therapeutic use of ESC transplantation in cardiac diseases primarily needs a rigorous demonstration that it can work in a stable fashion and with limited adverse effects.

Despite the limitations on federally funded research presently imposed, new sources of ESCs and cell lines for ESC transplantation studies need to be developed and likely will be, given the strong worldwide corporate- and state-funded interest in this technology and its purported benefits. Investigation into novel ways to isolate and culture autologous ESCs should also prove to be of significance.

Our overall understanding of the factors that may elicit the homing of ESCs to the heart and stimulate or direct the differentiation of ESCs to functional cardiomyocytes is presently rudimentary (a critique also applicable to adult stem cells). Identification of these factors and their mechanism of action will likely optimize both the homing

and the differentiation processes and will also to contribute to defining the best-case scenarios in which ESC transplantation will be beneficial.

12.1.7 Limitations and Concerns with Embryonic Stem Cell Transplantation

Considerable ethical and legal concerns about ESCs remain, and these concerns have significantly hampered further research efforts, which could provide needed cell lines as well as answers to many of the questions regarding the efficacy, long-term stability, function, and even the extent of the negative effects of ESC transplantation in cardiovascular disease. A concern often raised regarding the use of ESCs relates to their source (i.e., whether they originate from a cell line or directly from embryo), primarily heterologous versus autologous, posing the potential problem of generating an allogenic response or immunorejection upon transplantation. In addition, pluripotent ESCs that have unlimited growth potential can have tumorigenic side effects, making the screening for teratoma formation well advised. Moreover, evidence shows that differentiation of a heterogeneous ESC population is rather inefficient, although several agents (e.g., retinoic acid) appear to be effective in activating a greater extent of ESC-mediated cardiomyocyte-specific differentiation. The long-term stability of ESC-differentiated phenotype has also received mixed reviews because several studies have shown a loss of ESC=differentiated cardiomyocytes over time[5,28,29,54] (Table 12.4).

Transplanted ESC progeny may not always have a normal function because ESCs may promote arrhythmias in the transplanted hearts. On the other hand, the application of ESCs in repairing damaged, aging hearts may also be limited. This limitation has been proposed, but currently there is not solid data to support it. Nevertheless, cell transplants (either ESCs or adult stem cells) in the hearts of older individuals have frequently proved to be less effective. The inability of the damaged myocardium to provide the appropriate molecular signals for stem cells engraftment seems to limit their capacity for recruitment and integration into the aging myocardium (Coburn et al., 2005;[56–58]).

12.2 ADULT SKELETAL MYOBLAST CELLS

Transplanted satellite stem cells (myoblasts) from skeletal muscle can successfully home and engraft within a damaged myocardium, preventing progressive ventricular dilatation and improving cardiac function.[59] These myoblasts can be delivered into the myocardium by either intramural implantation or arterial delivery, and recently, effective deployment of a less invasive catheter approach has been reported. Skeletal muscle satellite cells can proliferate abundantly in culture and can be easily grown from the patients themselves (self-derived or autologous), thereby avoiding a potential immune response. Myoblasts are relatively ischemia resistant (compared with cardiomyocytes, which become injured within 20 min) because they can withstand several hours of severe ischemia without becoming irreversibly injured. The functional benefits of intramyocardially transplanted skeletal myoblasts in

TABLE 12.4 Different Types of Stem Cells for Cardiovascular Diseases

Cell Types	Advantages	Disadvantages
Embryonic stem cells	• Pluripotent and unlimited supply • Patient-specific cells for autologous transplantation possible via therapeutic donning	• Social and ethical concerns • Risk of rejection and required immunosuppression for allogenic transplant • Limited supply of human oocytes • Risk of tumor formation • Proarrhythmic risk because of immature phenotype of derived cardiomyocyte
Induced pluripotent stem cells	• Pluripotent and unlimited supply • Patient-specific cells for autologous transplantation possible	• Risk of tumor formation • Risk of viral vector • Proarrhythmic risk due to immature phenotype of derived cardiomyocyte
Skeletal myoblast	• Autologous transplantation without the need for immunosuppression or risk of rejection • Can be expanded *in vitro* with high yield, resistant to ischemia and fatigue	• Cannot differentiate into cardiomyocyte phenotype • Lack of integration with host cardiomyocyte with arrhythmogenic potential
Bone marrow stem cells	• Autologous transplantation without the need for immunosuppression or risk of rejection • Can induce angiogenesis; possibly pluripotent	• Limited ability to differentiate into cardiomyocyte • Limited supply and need for *in vitro* expansion • Difficult to isolate and propagate in culture
Mesenchymal stem cells	• Autologous transplantation without the need for immunosuppression or risk of rejection • Can induce angiogenesis and possible pluripotent • Lower risk of rejection and possibility for allogenic transplantation	• Limited ability to differentiate into cardiomyocyte • Limited supply and need for *in vitro* expansion • Difficult to isolate and propagate in culture
Adult cardiac stem cells	• Cardiomyocyte phenotype with no need for differentiation • Can integrate with host cardiomyocyte • Autologous transplantation without the need for immunosuppression or risk of rejection	• Very limited supply • Difficult to isolate and propagate in culture • Proarrhythmic risk because of immature phenotype of derived cardiomyocyte

Source: Adapted from Ref. [55].

improving the damaged myocardium secondary to ischemia have been well documented.

Initial clinical trials have shown the efficacy of autologous skeletal myoblast transplantation in patients with left ventricular (LV) dysfunction. The use of skeletal myoblasts delivered by multiple intramyocardial injections was effective in restoring LV function in a genetically determined Syrian hamster model of dilated cardiomyopathy, demonstrating that the functional benefits of transplanted skeletal myoblast can be extended to nonischemic cardiomyopathy.[59,60]

12.2.1 Advantages to Myoblast Transplantation

Because myoblasts can be of autologous origin and can be robustly expanded in culture, a large number of cells can be obtained from only a small skeletal muscle biopsy sample in a relatively short period of time. Compared with transplanted cardiomyocytes, myoblast cells appear to be more resistant to ongoing apoptotic damage, which tends to be prevalent at ischemic sites. Skeletal myoblasts were the first cells to be tried for cell-based cardiac therapy. They do not form tumors as with ESCs. Moreover, they can be easily handled and expanded *in vitro* (millions of myoblasts can be grown from a single muscle biopsy within a relatively short time). Myoblasts after injection into the infarcted heart have been shown to exhibit long-term engraftment;[61,62].

12.2.2 Disadvantages with Skeletal Myoblasts

Skeletal myoblasts do not adopt a cardiomyogenic differentiation .Moreover, they lack gap junctions, have not been shown to integrate electromechanically with the surrounding myocardium. They do not beat in synchrony and are isolated from the rest of the myocardium. Clinical trials of myoblast therapy have shown improvements in ejection fraction that persist 10 months following injection.[63,64]

12.2.3 Further Recommendations

Although preclinical studies with stem cell and myoblast transplantation have shown similar levels of efficacy, there is a need for a detailed evaluation on the relative benefits, adverse effects, and efficiency of skeletal myoblast and stem cell transplants in the clinical setting (e.g., heart failure) vis a vis the restoration of myocardial function. New methods to better assess and optimize posttransplanted myoblast recruitment and survival, particularly in the long term, need to be developed, and the repertoire of effective, less invasive cell delivery technologies needs to be expanded.

12.3 ADULT BONE MARROW–DERIVED STEM CELLS

The bone marrow contains a varied assortment of progenitor cells, including hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), multipotent

adult progenitor cells, and side population (SP) cells. Interest in bone marrow–derived stem cells has been mainly motivated by their neovascularization and angiogenesis properties, and these effects are enhanced by the presence of specific growth factors and cytokines (e.g., granulocyte colony-stimulating factor). Orlic and coworkers reported that after injection of bone marrow hematopoietic progenitors into the infarcted heart, the infarcted heart regenerated 68% of the myocardium and improved echocardiographic and hemodynamic indices of LV function.[65,66] Bone marrow progenitor cells evidently improve cardiac performance after myocardial injury.

It is important to point out that bone marrow contains several stem cell populations with overlapping phenotypes, including HSCs, endothelial stem/precursor cells (EPCs), mesenchymal stem cells (MSCs), and multipotent adult progenitor cells (MAPss). When endothelial progenitor cells originating from a common hemangioblast precursor in bone marrow, are delivered to the myocardial target area, they may implant, differentiate *in situ*, and promote new vessel growth, an approach that has been applied to several animal models of myocardial ischemia. These bone marrow–derived stem/precursor cells also can prevent the progression of cardiomyocytes, apoptosis, and stem cardiac remodeling.[65–69]

12.3.1 Advantages of Adult Bone Marrow Cell Transplantation

There is evidence that treatment with bone marrow cells (BMCs) can ameliorate both myocardial and vascular damage with increasing angiogenesis.[70] The effect of transplanted BMCs (which can include endothelial precursor cells) on vascular growth may significantly impact the recovery of the damaged heart (i.e., by improving oxygen availability), although this may depend on the myocardial setting, whether acute myocardial infarction or established heart failure. Moreover, autologous-derived cells for transplantation are an attractive alternative because bone marrow mesenchymal cells can be readily isolated in most cases. In addition, the expansion of BMC number by *in vitro* growth can be readily achieved by vigorous growth of mesenchymal cells in culture. It is significant that this method bypasses much of the ethical and legal concerns associated with the use of ESCs. Currently, it seems that MSCs likely exert their beneficial effects through the mechanisms pertaining to myocardial protection, ventricular remodeling, angiogenesis, and possibly myocyte regeneration.[71] The advantages are the immunotolerant properties stem cells (i.e., easy handling, their ability to home to injured myocardium after systemic delivery) have made them attractive tools for cardiac regenerative therapy (Beeres et al., 2003).

12.3.2 Limitations and Concerns with Adult Bone Marrow Cell Transplant

The mechanism of BMC-mediated augmentation of cardiomyocyte number and function remains controversial. Some studies have suggested that the effects of adult stem cell transplantation on the recipient heart are not a consequence of transdifferentiation but likely arise as a result of cell fusion with preexisting

cardiomyocytes or occur as a function of paracrine effects of transfected cells. Others maintain that there is evidence for a transdifferentiation event. Cell fusion has been demonstrated between cardiomyocytes and noncardiomyocytes *in vivo* and *in vitro*, and the data in support of transdifferentiation (particularly with HSCs) have not always been replicable. Further research is needed to clarify these issues and reconcile the contradictory claims as well as provide additional information about the extent of cell fusion and when it occurs. A limitation of the majority of the clinical studies with adult noncardiac stem cell transplantation relates to the potential stability of the differentiated phenotype because these studies have primarily examined the short-term benefits.[72]

12.3.3 Resident Cardiac Progenitor Cells

Cardiac progenitor cell are capable of multilineage differentiation. Beltrami and coworkers[73] were the first to describe the existence of a resident cardiac progenitor cell (CPC) capable of differentiating into myocytes as well as endothelial and smooth muscle cells. It has been shown that when these resident cardiac progenitor cells are injected into the postinfarct heart, there is some improvement of cardiac function.[74]

12.3.4 Adult Stem Cells

Adult or somatic stem cells exist throughout the body after embryonic development and are found inside of different types of tissue. These stem cells have been found in tissues such as the brain, bone marrow, blood, blood vessels, skeletal muscles, skin, and liver. They remain in a quiescent or nondividing state for years until activated by disease or tissue injury. They are multi(pluri)potent.[73,75]

Adult stem cells are unique cells that can divide or self-renew indefinitely, enabling them to generate a range of cell types from the originating organ or even regenerate the entire original organ. They have the ability to differentiate through a committed lineage. It is generally thought that adult stem cells are limited in their ability to differentiate based on their tissue of origin, but some evidence suggests that they can differentiate to become other cell types. These adult stem cells comprise at least three different groups: bone marrow–derived stem cells, the circulating pool of stem or progenitor cells, and tissue resident stem cells Adult stem cells typically generate the cell types of the tissue in which they reside (e.g., a blood-forming adult stem cell in the bone marrow), and they normally gives rise to the many types of blood cells such as red blood cells, white blood cells, and platelets. Stem cells from one type of tissue may be able to give rise to cell types of a completely different tissue; that is, they exhibit plasticity (e.g., blood cells becoming neurons and heart muscle, liver cells that can be made to produce insulin).

Stem cells, depending on their lineage commitment, possess the ability to differentiate into cells of various tissues; this property of stem cell is called differentiation. Embryonic cells are pluripotent and can generate tissues belonging to all three germ layers. Adult stem cells are thought to be more committed and

possess a limited ability to differentiate along a specific lineage. In contrast to ESCs, differentiation of adult bone marrow stem cells into functional cardiomyocytes has been more difficult to demonstrate. *Transdifferentiation* is a term used to define a committed stem cell crossing lineage boundaries and differentiating into cells belonging to another lineage. An HSC giving rise to cardiomyocytes is an example of transdifferentiation. *Fusion* refers to the phenomenon in which stem cells fuse with somatic cells; the resultant hybrid cells usually assume the more undifferentiated phenotype but possess some characteristics of both cell types. For example, bone marrow cells, when grown in culture with ESCs, could fuse with ESCs and adopt the recipient phenotype. Stem cells can regulate tissue regeneration and repair. Apart from angiogenesis and wound healing, stem cells could exert a host of other paracrine effects on myocardial protection, cardiac contractility, myogenic differentiation of resident cardiac progenitors, and scar formation.[19,76]

12.3.5 Advantages of Adult Stem Cells

Although the implantation of skeletal myoblasts and adult BMC transplantation appears promising, adult stem cell transplantation might be more effective than adult BMC transplantation because cardiac stem cells may be better programmed. The further identification, purification, and characterization of the adult stem cells as well as a detailed knowledge of their interactions with the cardiac milieu or niche are essential if we are to achieve the major goal of regenerating or transplanting the tissue to treat myocardial infarction.

12.3.6 Limitations of Adult Stem Cells

Until recently, data on the presence of adult stem cells have been scarce. This subset of stem cells appears to be extremely limited in number and difficult to identify and expand in culture, thereby limiting their characterization and utilization and likely contributing to difficulties in reproducing experiments concerning their isolation and transplantation. In addition, there is presently no consensus regarding the definition of selective markers specific for adult stem cell type.

12.3.7 Culturing Embryonic Stem Cells in the Laboratory

Human ESCs are isolated by transferring the inner cell mass to a plastic laboratory culture dish that contains a nutrient broth known as culture medium. The cells divide and spread over the surface of the dish. The inner surface of the culture dish is typically coated with mouse embryonic skin cells that have been treated so they will not divide. This coating layer of cells is called a *feeder layer*. This layer provides a sticky surface for the inner cell mass to which they can attach. Also, the feeder cells release nutrients into the culture medium. Culture media without the feeder cell layer is also available, so there are no chances of transmission of viral disease. ESCs that have proliferated in cell culture for 6 or more months without differentiating, are

pluripotent, and appear genetically normal are referred to as an ESC line.[77] After cell lines are established, or even before that stage, batches of them can be frozen and shipped to other laboratories for further culture and experimentation. The process of growing large numbers of ESCs has been easier than growing large numbers of adult stem cells, but progress is being made for both cell types.

12.3.8 Stem Cell Lines

After stem cells have been allowed to divide and propagate in a controlled culture, the collection of healthy, dividing, and undifferentiated cells is called a stem cell line. These stem cell lines are subsequently managed and shared among researchers. When under control, the stem cells can be stimulated to specialize as directed by a researcher, a process known as directed differentiation. ESCs are able to differentiate into more cell types than adult stem cells.[78]

12.3.9 Tests Used to Identify Embryonic Stem Cells

During the process of generating embryonic stem cell lines to see whether they exhibit the fundamental properties that makes them embryonic stem cells, various tests are carried out, this process is called *characterization*. The process includes growing and subculturing the stem cells for many months. This ensures that the cells are capable of long-term self-renewal. Microscopy used to see that the cells look healthy and remain undifferentiated. Surface markers are found only on undifferentiated stem cells.[77]

12.3.10 Tests Used in Identifying Adult Stem Cells

Labeling the cells in a living tissue with molecular markers and then determining the specialized cell types they generate, removing the cells from a living animal, labeling them in cell culture, and transplanting them back into another animal to determine whether the cells repopulate their tissue of origin. Isolating the cells, growing them in cell culture, and manipulating them, often by adding growth factors or introducing new genes, to determine what differentiated cells types they can become.

12.4 TYPE OF STEM CELLS USED TO TREAT CARDIAC DISEASES

A brief comparison of the advantages and limitations of the cell types presently used in cardiac transplantation is shown in Table 12.5. Although no clear-cut choice has yet emerged as to which cell type is best to transplant in myocardial repair, there are reasons to believe that the development of a multiplicity of approaches in the application of cell engineering will be required to develop novel therapies for different cardiac disorders.

The approach to treat heart failure may require the transplantation of cell types (e.g., skeletal myoblasts) that are different than those used in the targeted treatment of cardiac arrhythmias, conduction disorders, and congenital defects. It is also

TABLE 12.5 Advantages and Disadvantages of Potential Clinical Strategies for Stem Cell Delivery

Strategies	Potential Clinical Applications	Clinical Experience	Advantages	Disadvantages
Intravenous	Acute MI	+/−	• Easy available and avoids the risk of any invasive procedure	• Low efficacy of cell delivery • Not applicable to patients with an occluded • Risk of systemic administration unclear
Intracoronary	Acute MI Chronic MI	+++ +	• Possible wide use in catheterization laboratory • Limited risk of systemic administration • Clinical trials ongoing	• Efficacy of cell delivery to the myocardium uncertain • Not applicable to patients with a occluded artery • Not applicable for stem cell with large cells
Catheter-based direct intramyocardial injection	Chronic myocardial ischemia Acute MI	+++ +	• Avoid the risk of open heart surgery • Higher efficacy of cell delivery • Short-term safety proven • Clinical trials ongoing	• Need for specialized catheters and imaging technology to guide the procedure
Open heart direct epicardial injection	Chronic myocardial ischemia Chronic MI	++ ++	• Applicable to patients who need open heart surgery • Allows direct visualization of the site of injection	• Risk of mortality and morbidity of open heart surgery
Direct epicardial patch	Chronic MI	–	• Applicable to patients who need open heart surgery • Allows uneven distribution of cell in the myocardium • Allows direct delivery of a large amount of cell	• Needs tissue engineering to create cellular patch • Risk of mortality and morbidity of open heart surgery

Source: Adapted from Ref. [80].

possible that the long-term repair of a fully functioning myocardium may require more than a single cell type (e.g., cardiomyocytes, fibroblasts, and endothelial cells) in the generation and integration of a stable and responsive cardiac graft.[13,30,31,84–87]

12.4.1 Potency

Stem cells are categorized by their potential to differentiate into other types of cells. ESCs are the most potent because they must become every type of cell in the body. The full classification includes totipotent, pluripotent, multipotent, oligopotent, and unipotent. *Totipotent* is the ability to differentiate into all possible cell types. Examples are the zygote formed at egg fertilization and the first few cells that result from the division of the zygote. *Pluripotent* is the ability to differentiate into almost all cell types. Examples include ESCs and cells that are derived from the mesoderm, endoderm, and ectoderm germ layers that are formed in the beginning stages of ESC differentiation. *Multipotent* is the ability to differentiate into a closely related family of cells. Examples include hematopoietic (adult) stem cells that can become red and white blood cells or platelets. *Oligopotent* is the ability to differentiate into a few cells. Examples include (adult) lymphoid or myeloid stem cells. *Unipotent* is the ability to only produce cells of their own type but having the property of self-renewal required to be labeled a stem cell. Examples include (adult) muscle stem cells. ESCs are considered pluripotent instead of totipotent because they do not have the ability to become part of the extraembryonic membranes or the placenta.

12.4.2 Identification of Stem Cells

Although there is not complete agreement among scientists of how to identify stem cells, most tests are based on making sure that stem cells are undifferentiated and capable of self-renewal. Tests are often conducted in the laboratory to check for these properties. The standard procedure for testing bone marrow or HSCs is by transplanting one cell to save an individual without HSCs. If the stem cell produces new blood and immune cells, it demonstrates its potency. Clonogenic assays (a laboratory procedure) can also be used *in vitro* to test whether single cells can differentiate and self-renew. Researchers may also inspect cells under a microscope to see if they are healthy and undifferentiated, or they may examine chromosomes. To test whether human ESCs are pluripotent, scientists allow the cells to differentiate spontaneously in cell culture, manipulate the cells so they will differentiate to form specific cell types, or inject the cells into an immunosuppressed mouse to test for the formation of a teratoma (a benign tumor containing a mixture of differentiated cells).

12.4.3 Mechanisms of Action of Stem Cells

A co-culture technique was developed whereby stem or progenitor cells are cultured together with rat neonatal ventricular cardiomyocytes to simulate a cardiac like environment *in vitro*. The neurohormone oxytocin or cytokines of the Wnt or

platelet-derived growth factor (PDGF) family have been used to induce or enhance differentiation of adult stem cells. The identification of subsets of adult stem cells with a higher capacity to differentiate into cardiac myocytes is currently under investigation. Progenitor cells may improve neovascularization and thereby augment nutrients and oxygen supply. Neovascularization can be mediated by the physical incorporation of progenitor cells into new capillaries or by perivascular accumulation of cells. Incorporated progenitor cells of most, if not all, types may release growth factors that promote angiogenesis by acting on mature endothelial cells. Paracrine factors may also beneficially influence cardiac repair by protecting cardiomyocytes from apoptotic stimuli or activate cardiac resident stem cells to enhance the endogenous repair capacity. The release of various cytokines affects the cardiac remodeling processes by altering the development of fibrosis development during scar formation or by modulating inflammatory processes .The extent to which progenitor cells contribute to vasculogenesis depends on the environment to which the cells are exposed. Thus stem cells mechanisms to be considered include transdifferentiation of stem cells, enhanced neovascularization, alterations in scar formation, and cytoprotection.

12.4.4 Immunomodulatory Effect of Stem Cells

Numerous studies have demonstrated that human mesenchymal stem cells (hMSCs) avoid allorecognition, interfere with dendritic cell and T-cell function, and generate a local immunosuppressive microenvironment by secreting cytokines. It has also been shown that the immunomodulatory function of human MSCs is enhanced when the cells are exposed to an inflammatory environment characterized by the presence of elevated local interferon-γ (INF-γ) levels. Other studies contradict some of these findings, reflecting both the highly heterogeneous nature of MSC isolates and the considerable differences between isolates generated by the many different methods under development.

Mesenchymal stem cells are multipotential nonhematopoietic progenitor cells capable of differentiating into multiple mesenchymal tissues. hMSCs are characterized by a low expression of major histocompatibility complex (MHC) class I and the absence of co-stimulatory molecules such as CD80, CD86, or CD40. Moreover, hMSCs fail to induce proliferation of allogeneic or xenogeneic lymphocytes.

These characteristics support the possibility of exploiting universal donor MSC for therapeutic applications. MSCs constitutively express low levels of MHC-I molecules, but, as a general rule, they do not constitutively express MHC class II molecules However, recent evidence indicates that MSC can function as antigen-presenting cells and activate immune responses under appropriate conditions. Although one study reported constitutive MHC class II expression on MSC, several groups reported that both MHC class I and class II molecules are upregulated after IFN-γ treatment, thus inducing a T-cell response to recall antigens.

Mesenchymal stem cells have an immunomodulatory effect, which is currently being exploited in the clinical setting for the treatment of coronary artery diseases.

12.4.4.1 Drugs That Have Immunomodulatory Effects on T Cells and Dendritic Cells A new group of tyrosine kinase inhibitors, exemplified by the Bcr-Abl inhibitor imatinib, avoids the side effects of systemic chemotherapies and the high morbidity and mortality risks associated with HSC transplantation. Concurrently, however, increasing evidence has emerged to indicate that these drugs exert profound immunomodulatory effects on T cells and antigen-presenting cells, such as dendritic cells, that play major roles in immune tumor surveillance and the outcome of HSC transplantation. Targeted tyrosine kinase inhibitor therapy may thus control cancer cell growth both directly and indirectly by changing the immunologic microenvironment. Furthermore, such molecules might help to unravel the complexities of the human infectious processes.

12.4.4.2 Immunomodulatory Properties of Mesenchymal Stem Cells Derived from Dental Pulp and Dental Follicle are Susceptible to Activation by Toll-Like Receptor Agonists Adult MSCs have recently become a potent tool in regenerative medicine. Because of certain shortcomings of obtaining bone marrow MSCs, alternate sources of MSCs have been sought. MSCs from dental pulp (DP-MSCs) and dental follicle (DF-MSCs), isolated from the same tooth or donor, to define differences in their phenotypic properties, differentiation potential, and immunomodulatory activities. Both cell types showed colony-forming ability and expressed typical MSCs markers but differed in the levels of their expression. DF-MSCs proliferated faster, contained cells larger in diameter, and exhibited a higher potential to form adipocytes and a lower potential to form chondrocytes and osteoblasts compared with DP-MSCs.

In contrast to DF-MSCs, DP-MSCs produced TGF-β and suppressed proliferation of peripheral blood mononuclear cells (PBMCs), which could be neutralized with anti–TGF-β antibody. The treatment with Toll-like receptor 3 (TLR3) agonist augmented the suppressive potential of both cell types and potentiated TGF-β and interleukin-6 secretions by these cells. TLR4 agonist augmented the suppressive potential of DF-MSCs and increased TGF-β production but abrogated the immunosuppressive activity of DP-MSCs by inhibiting TGF-β production and the expression of indolamine-2,3-dioxygenase-1. Some of these effects correlated with the higher expression of TLR3 and TLR4 by DP-MSCs compared with DF-MSCs. Dental MSCs are functionally different, and each of these functions should be further explored *in vivo* before their specific biomedical applications are used.

12.5 APPLICATION

12.5.1 Routes of Application

Progenitor cells for cardiac repair can be delivered in different ways via the intracoronary route or by direct injection into the myocardium using a percutaneous catheter-based or surgical epicardial approach. Intracoronary infusion using standard balloon catheters has been used in all clinical trials treating patients with acute

myocardial infarction. The advantage is that cells can travel directly only into myocardial regions in which nutrient blood flow and oxygen supply are preserved, thereby ensuring a favorable environment for cell survival, a prerequisite for stable engraftment.

Trials using the intracoronary approach have administered the cells in the culprit artery from 5 to 14 days after MI. Direct intracoronary injection after revascularization has the obvious advantage of the cells reaching previously underperfused regions of the myocardium. Potentially, the perfused myocardium also creates a more suitable environment for engraftment of the progenitor cells. In the heart, less perfused regions of the myocardium receive fewer cells; thus, this route may be inefficient for successful targeting of underperfused myocardium, an important consideration for patients with extensive microvascular disease. The type of stem cell administered is another important consideration for selecting the route of administration.[88–95]

Skeletal myoblasts are larger cells and may even obstruct the microcirculation and lead to greater injury. A decrease in distal blood flow and embolic risk may be clinically significant, especially if the cells are administered at the time of primary revascularization after MI. Direct injection of stem cells into the heart may obviate the problems of decreased uptake of cells in less perfused regions of the myocardium but runs risks of cardiac perforation. Moreover, the necrotic, hypoxic, and inflamed myocardium, into which the cells are directly injected, may not provide the cells with the best microenvironment for effective tissue repair. Intraarterially, homing of intraarterially applied progenitor cells requires migration out of the vasculature into the surrounding tissue, which may mean that unperfused regions of myocardium will be targeted far less efficiently, if at all. Bone marrow–derived and blood-derived progenitor cells are known to extravasate and migrate to ischemic areas. Other cell types may not, and they may even obstruct the microcirculation, which leads to embolic myocardial damage. Clinical trials of cardiac cell therapy suggest that injection of stem cells at least 4 days after myocardial injury leads to improvement in ejection fraction compared with earlier injection. Focal injection of stem cells may not be the optimal route in diseases that affect the myocardium more globally such as nonischemic dilated cardiomyopathy.

12.5.2 Complications

The risk for ventricular perforation is high. Most cells, if injected directly, simply die. In diffuse disease such as dilated nonischemic cardiomyopathy because focal deposits of directly injected cells might be poorly matched to the underlying anatomy and physiology, so additional strategies to augment cell homing and to promote homogeneous integration of cells may be required.

12.5.3 Using Stem Cells in Clinical Application and to Treat Disease

Currently, a variety of autologous adult progenitor cells are undergoing clinical evaluation. The first clinically relevant cells proposed for cardiac myocytes were

skeletal muscle myoblasts, undifferentiated proliferation competent cells that serve as precursors to skeletal muscle. For clinical use, autologous human myoblasts are isolated from skeletal muscle biopsies, propagated and expanded *ex vivo* for a few days or weeks, and then injected directly into the ventricular wall. Bone marrow is, at present, the most frequent source of stem cells used clinically for cardiac repair. Bone marrow is aspirated under local anesthesia in most of the studies; the entire mononuclear cell fraction is obtained. Isolated bone marrow–derived cells have been injected into the heart without further *ex vivo* expansion. These circulating "EPCs" are the basis for virtually all clinical studies that have used bone marrow or its circulating derivatives for the treatment of ischemic myocardium.[8,9,11,14,15,30,31,43,55,57,96–98]

The first step in using stem cells for disease treatment is to establish stem cell lines, which researchers have accomplished. Next, scientists must be able to turn on specific genes within the stem cells so that the stem cells will differentiate into any cell they wish. But scientists have not learned how to do this yet, so studying stem cell differentiation is an active area of research. When scientists are able to create differentiated cells from stem cells, then there are many possibilities for their use, such as drug testing and cell-based therapies. For example, let us say you want to test new drugs to treat heart diseases. Currently, new drugs must be tested on animals. The data from animal research must be interpreted and then extrapolated to humans before human clinical trials. But suppose you could test them directly on human heart cells. To do this, human stem cell lines could be treated to differentiate into human heart cells in a dish. The potential drugs could be tested on those cells, and the data would be directly applicable to humans. This use could save vast amounts of time and money in bringing new drugs to market.

Stem cell–based therapies are not new. The first stem cell–based therapy was a bone marrow transplant used to treat leukemia. In this procedure, the patient's existing bone marrow is destroyed by radiation, chemotherapy, or both. Donor bone marrow is injected into the patient, and the bone marrow stem cells establish themselves in the patient's bones. The donor bone marrow cells differentiate into blood cells that the patient needs. Often, the patient must take drugs to prevent his or her immune system from rejecting the new bone marrow. But this procedure uses existing hemopoietic stem cells. How would you use stem cell lines? Let us look at how stem cells might be used to treat heart failure.

Ideally, to treat a failing heart, scientists could stimulate stem cells to differentiate into heart cells and inject them into the patient's damaged heart. There, the new heart cells could grow and repair the damaged tissue. Although scientists cannot yet direct stem cells to differentiate into heart cells, they have tested this idea in mice. They have injected stem cells (adult, embryonic) into mice with damaged hearts. The cells grew in the damaged heart cells, and the mice showed improved heart function and blood flow. In these experiments, exactly how the stem cells improved heart function remains controversial. They may have directly regenerated new muscle cells. Alternatively, they may have stimulated the formation of new blood vessels into the damaged areas. And the new blood flow may have stimulated existing heart stem cells to differentiate into new heart muscle cells. These experiments are currently being evaluated.

One major obstacle in stem cell use is the problem of rejection. If a patient is injected with stem cells taken from a donated embryo, his or her immune system may see the cells as foreign invaders and launch an attack against them. Using adult stem cells or induced pluripotent stem cells (IPSCs) could overcome this problem somewhat because stem cells taken from the patient would not be rejected by his or her immune system; however, the adult stem cells are less flexible than ESCs and are harder to manipulate in the laboratory.[99] IPSC technology is too new for transplantation work.[100]

Finally, by studying how stem cells differentiate into specialized cells, the information gained can be used to understand how birth defects occur and possibly how to treat them. So, if there is so much potential in stem cell research, why all the controversy? Let us investigate the current ethical and political issues.[5,27,28,29,38,56,71,101–111]

12.5.4 Results of Clinical Trials

In patients with acute myocardial infarction, progenitor cell transplantation aims to prevent or ameliorate postinfarction LV remodeling, thereby reducing postinfarction HF. Such an effect might be achieved by enhanced neovascularization and reduced cardiomyocyte apoptosis, irrespective of long-term engraftment and transdifferentiation. In patients with chronic HF, cardiomyogenesis in its pure sense would be desirable.[58,85,91,96,112–115]

12.5.5 Cell Therapy in Acute Myocardial Infarction

Infusion of autologous bone marrow mononuclear cells (BMCs) is safe and feasible in patients with acute myocardial infarction, which is supported by the TOPCARE-AMI, the BOOST trial, which showed there is improvement in global LV ejection fraction by 7% to 9%, and there is significant reduction in LV end-systolic volume, which has improved perfusion in the infarcted area in 4–6 months after cell transplantation. Whereas the randomized, controlled trial by Janssen did not reveal a significant effect on global ejection fraction, it did show an increase in regional ejection fraction and a reduction of the infarct size in the BMC group. Another trial, ASTAMI (Autologous Stem Cell Transplantation in Acute Myocardial Infarction), did not show any benefit.

Overall, the clinical data available indicate that cell therapy with bone marrow–derived cells is feasible and safe at least for the follow-up presently available (up to 5 years in the pioneering studies). None of the studies so far reported an increased incidence of arrhythmias (as has been seen in myoblast trials). Moreover, restenosis, which was considered as a potential side effect by progenitor cell–mediated plaque angiogenesis or plaque inflammation, was increased only in one study using $CD133^+$ cells, Because $CD133^+$ cells were isolated by using a mouse antibody, one may speculate that the remaining antibody might have elicited a local proinflammatory reaction. All other studies did not observe an augmented risk for restenosis; if anything, there was a significantly decreased necessity for revascularization

procedures in the REPAIR-AMI trial. Careful evaluation of the 18-month follow-up data of the BOOST trial indicates that the ejection fraction of the cell therapy group is maintained from 6 to 18 months of follow-up. The long-term 2 year follow-up MRI-derived data of the TOPCARE-AMI trial showed that the ejection fraction is maintained and even further augmented in the treated patients, in parallel with a sustained reduction in NT pro-BNP serum levels, suggesting a beneficial effect of long-term LV remodeling. Taken together, these data may provide the rationale to assess the effects of intracoronary BMC infusion on clinical outcome in a large patient cohort with severe acute myocardial infarction.[14,111,116–126]

12.5.6 Research with Stem Cells

Scientists and researchers are interested in stem cells for several reasons. Although stem cells do not serve any one function, many have the capacity to serve any function after they are instructed to specialize. Every cell in the body, for example, is derived from first few stem cells formed in the early stages of embryologic development. Therefore, stem cells extracted from embryos can be induced to become any desired cell type. This property makes stem cells powerful enough to regenerate damaged tissue under the right conditions.

12.5.7 Organ and Tissue Regeneration

Tissue regeneration is probably the most important possible application of stem cell research. Currently, organs must be donated and transplanted, but the demand for organs far exceeds supply. Stem cells could potentially be used to grow a particular type of tissue or organ if directed to differentiate in a certain way. Stem cells that lie just beneath the skin, for example, have been used to engineer new skin tissue that can be grafted on to burn victims.[127,128]

12.5.8 Brain Disease Treatment

Additionally, replacement cells and tissues may be used to treat brain disease such as Parkinson's and Alzheimer's diseases by replenishing damaged tissue, bringing back the specialized brain cells that keep unneeded muscles from moving. ESCs have recently been directed to differentiate into these types of cells, so treatments are promising.[129–131]

12.5.9 Cell Deficiency Therapy

Healthy heart cells developed in a laboratory may one day be transplanted into patients with heart disease, repopulating the heart with healthy tissue. Similarly, people with type I diabetes may receive pancreatic cells to replace the insulin-producing cells that have been lost or destroyed by the patient's own immune system. The only current therapy is a pancreatic transplant, and it is unlikely to occur because of a small supply of pancreases available for transplant.[130,132–136]

12.5.10 Blood Disease Treatments

Adult HSCs found in blood and bone marrow have been used for years to treat diseases such as leukemia, sickle cell anemia, and other immunodeficiencies. These cells are capable of producing all blood cell types, such as red blood cells that carry oxygen to white blood cells that fight disease. Difficulties arise in the extraction of these cells through the use of invasive bone marrow transplants. However, HSCs have also been found in umbilical cords and placentas. This has led some scientists to call for an umbilical cord blood bank to make these powerful cells more easily obtainable and to decrease the chances of a body's rejecting therapy.[137,138]

12.5.11 General Scientific Discovery

Stem cell research is also useful for learning about human development. Undifferentiated stem cells eventually differentiate partly because a particular gene is turned on or off. Stem cell researchers may help to clarify the role that genes play in determining what genetic traits or mutations we receive. Cancer and other birth defects are also affected by abnormal cell division and differentiation. New therapies for diseases may be developed if we better understand how these agents attack the human body. Another reason why stem cell research is being pursued is to develop new drugs. Scientists could measure a drug's effect on healthy, normal tissue by testing the drug on tissue grown from stem cells rather than testing the drug on human volunteers.[139,140]

12.5.12 Transplantation and Left Ventricular Devices

Left ventricular devices to an earlier stage of heart failure when candidates with the highest risk can be avoided. By ratcheting back on the severity of illness, it is believed that perioperative mortality rate and complications will be reduced to a minimum, and the treatment will be a test of the pump's reliability and biocompatibility versus the best available medical therapy. A number of micropumps are under development to target less ill patients. They are designed for implantation either by a minimally invasive surgical procedure or, remarkably, by interventional catheter-based techniques. Their small size places the optimal rates of flow in the range of 2–3 l/min. These devices, unlike larger pumps that replace LV function, are designed to assist and more correctly are defined as ventricular assist devices. One can only conjecture as to whether these types of devices will reduce progression of heart failure or even reverse remodel early-stage disease.

12.6 OTHER DEVELOPING TECHNOLOGIES IN CELL ENGINEERING

12.6.1 Hybrid Embryos

British scientists plan to create the world's first human stem cells from embryos that are part human and part animal.

Human skin cells will fuse with empty pig eggs to create embryos that contain 99.9% human DNA and 0.1% pig DNA. Stem cells extracted from the embryos will then be treated with chemicals to destroy the pig DNA before they are grown into human heart cells. The animal DNA is destroyed to make the cells behave more like human cells.

This will represent a landmark in stem cell science and give researchers a way to make almost unlimited stocks of human ESCs, which in principle can grow into any tissue in the body. Scientists have so far been unable to create stem cells using human eggs, which are in short supply.

Although the stem cells will not contain any animal DNA, they will not be suitable for treating humans directly. Instead, the scientists will use the cells to learn how genetic mutations cause heart cells to malfunction and ultimately cause life-threatening cardiomyopathy. Ultimately, they will help us understand where some of the problems associated with these diseases arise, and they could also provide models for the pharmaceutical industry to test new drugs.

12.6.2 Upcoming Techniques in Guidance to Homing of Stem Cell

Adipose tissue is another rich source of distinct subsets of stem and progenitor cells that are potentially useful for cardiac repair and neovascularization improvement. Both mesenchymal stem cells and endothelial progenitor cells have been isolated after enzymatic digestion of adipose tissue and showed beneficial effects in experimental studies. Very recently, pluripotent spermatogonial stem cells from adult mouse testis that possess the capacity to differentiate to fully active cardiac myocytes *in vitro* have been identified.

In diffuse disease such as dilated nonischemic cardiomyopathy, focal deposits of directly injected cells might be poorly matched to the underlying anatomy and physiology. Thus, it is likely that the nature of the patient's cardiomyopathy will ultimately influence, if not dictate, the source and route chosen among potential progenitor cell therapies. Intravenous administration of cells may be hampered by trapping of the cells in the pulmonary circulation. Indeed, in clinical trials with labeled bone marrow–derived cells, no homing to the heart in acute myocardial infarction was observed after intravenous cell administration. However, intravenous application of allogeneic mesenchymal stem cells was used safely and is currently being tested in a clinical phase II study.

Randomized controlled trials currently assessing the effects of intracoronary administration of BMCs in patients with acute myocardial infarction: are the REGENT (Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction), FINCELL (Effects of Intracoronary Injection of Mononuclear Bone Marrow Cells on LV Function, Arrhythmia Risk Profile, and Restenosis After Thrombolytic Therapy of Acute Myocardial Infarction), and ASTAMI (Autologous Stem Cell Transplantation in Acute Myocardial Infarction) studies (Tables 12.6–12.8).

Three of the trials were placebo controlled. The primary end point common to all these trials was change in LVEF at 4 to 6 months. In four of the trials, recovery of global LVEF was significantly greater in the BMC-treated patient group compared

TABLE 12.6 Randomized Controlled Trials in Acute Myocardial Infarction

	No. of Patients	Cell Types and Numbers ($\times 10^6$)	Time to Therapy (Days)	Follow-up Duration (Months)	Primary End Point	LVEF (%)	Infarct Size	Side Effects
BOOST-2 trial	33 versus 30 controls	2460 BMC with gelatine–polysuccinate density gradient (9.5 $CD34^+$)	4.8	18	LVEF (MRI), safety	6 months: +6.7% versus +0.7% (↑ 6%) 18 months: +5.9% versus +3.1% (NS)	NS	Nil
Jannsens et al.	33 versus 34 controls	304 BMC with Ficoll (2.8 $CD34^+$)	1	4	LVEF (MRI)	+3.4% versus +2.2% (NS)	↓	Nil
ASTAMI	50 versus 50 controls	68 BMC with Lymphoprep Ficoll (0.7 $CD34^+$)	6	6	LVEF infarct size (MRI, SPECT, ECHO)	+1.2% versus +4.3% (NS)	NS	Nil
MAGIC CELL-3-DES	25 versus 25 controls	1500 G-CSF mobilized PBC (7×10^6 $CD34^+$)	4	6	LVEF (MRI)	+5.5% versus 0% (↑ 5.5%)	↓	Nil
REPAIR-AMI	95 versus 92 controls	236 BMC with Ficoll (3.6 $CD34^+$)	4.4	12	LVEF (LV angiography)	5.5% versus + 3.0% (↑ 2.5%)	NS	Nil
Meluzin et al.	44 (high and low) versus 22 controls	High: 100 BMC Low: 10 BMC	7	3	LVEF (SPECT)	High: +5%; low: +3% Controls: 2% (↑2.0%)	NS	Nil
REGENT	117 (selected and unselected) versus 20 controls	Unselected: 178 BMC Selected: 1.9 $CD34/CXCR4^+$	7	6	LVEF (MRI)	Unselected +3% Selected: +3% Control: 0% (NS)	NA	Nil
FINCELL	40 versus 40 controls	402 BMC	2–6	12	LVEF (ECHO)	+7.1% versus +1.2%	NA	Nil

BMC, bone marrow cells; g-CSF, granulocyte colony simulating factor; PBC, peripheral blood cells; LVEF, left ventricular ejection fraction; MRI, magnetic resonance imaging; NA, not available; NS, non-significant.
Source: Adapted from Ref. [80].

TABLE 12.7 Randomized Controlled Trials in Chronic Myocardial Ischemia

	No. of Patients	Cell Types and Numbers ($\times 10^6$)	Follow-up Duration (Months)	Primary End Points	Secondary End Points	Side Effects
Losordo et al.	18 versus 6 controls	0 05–0.5 per kg G-CSF mobilized PBC	6	Anginal frequency: −12 6 versus −4.5 (↓8.1 NS) Safety: NS	SPECT perfusion score −1.5 versus −2.2 (NS) Exercise time: +0.5 versus +0.3 min (NS)	Nil
PROTECT-CAD	19 versus 9 controls	42 BMC with Ficoll (1.38 $CD34^+$)	6	Exercise time: ↑53%	LVEF (MRI): +3.7% versus −0.4% (↑5.4%) SPECT perfusion score: −0.5 versus +2.4 (↓2.7, NS)	Nil
Van Ramshorst et al.	25 versus 25 controls	98 BMC with Ficoll	6	SPECT perfusion score: −3.4 versus −1.1 (↓2.44)	LVEF (MRI): +3% versus −1% (↑3%) Exercise capacity: +9W versus +2W (↑7W)	Nil

Source: Adapted from Ref. [80].

TABLE 12.8 Randomized Controlled Trials in Congestive Heart Failure

	No. of Patients	Cell Types and Numbers ($\times 10^6$)	Follow-up Duration (Months)	Primary End Point	LVEF (%)	LV Dimension	Side Effects
TOPCARE-CHD	24 (PBC), 24 (BMC) versus 23 (controls)	Intracoronary injection: 22 PBC or 205 BMC with Ficoll	3	LVEF (LV angiogram)	PBC: −0.4%; BMC: +2 9%; control: −1.2%	LV EDV-PBC: −3%; BMC: 0%; control: −3% (NS) LVESV-PBC: −2%; BMC: +2%; control: −1% (NS)	Nil
MAGIC	63 high and low dose versus 34 controls	Epicardial injection: 400 skeletal myoblast (low dose); 800 skeletal myoblast (high dose)	6	LVEF and changes in number of akinetic segments (ECHO)	High dose: +5.2% Low dose: +3.4% Control: +4.4% (NS)	High: −12.6 Low: −3.9 Control: +5.9 (↓ 12.8) LV EDV (ml/m)- High: −8.3 Low: −6.5 Control: −2.1 (↓ 8.1)	NS higher number of arrhythmic events in myoblast group

LV, left ventricular; ESV, end-systolic volume; EDV, end-diastolic volume.
Source: Adapted from Ref. [80].

with the placebo or control group; one trial demonstrated only regional contractile improvement in the infarcted segments, and one trial did not show any differences between the treatment and the control groups. The Leuvin-AMI trial, which showed only regional contractile improvement in the infarcted segment, differed importantly from the other trials with respect to the timing of BMC administration, which was performed within 24 hours after reperfusion therapy for acute myocardial infarction. Although not yet published in manuscript form, the results of the HEBE trial (Bone Marrow Cell Therapy After Acute Myocardial Infarction) have been presented recently in abstract form.

In the HEBE trial, 200 patients with acute myocardial infarction were randomly assigned either to receive an infusion of mononucleated BMCs or mononucleated cells isolated from PBMCs or to primary percutaneous angioplasty alone (1:1:1 ratio). Despite promising results in the pilot trial, intracoronary infusion of mononucleated BMCs or PBMCs did not improve regional LV systolic function (primary end point) or global LV function and LV remodeling (secondary end points) at 4 months, assessed by MRI. The reasons that the ASTAMI and HEBE trials failed to show a benefit of cell therapy are unclear. However, preclinical work suggested that the processing techniques used to isolate the cells may have affected the outcome of the ASTAMI trial. The reasons for these negative findings of the HEBE trial are unclear and will remain speculative until publication of the full trial.

Stem cell researchers use the signaling molecules that selectively adhere to the receptors on the surface of the cell as a tool that allows them to identify stem cells. Many years ago, a technique was developed to attach to the signaling molecule another molecule (or the tag) that has the ability to fluoresce or emit light energy when activated by an energy source such as an ultraviolet light or laser beam.

There are two approaches of how researchers use the combination of the chemical properties of fluorescence and unique receptor patterns on cell surfaces to identify specific populations of stem cells. One approach for using markers as a research tool is with a technique known as fluorescence-activated cell sorting (FACS).

A second method uses stem cell markers and their fluorescent tags to visually assess cells as they exist in tissues. Often researchers want to assess how stem cells appear in tissues, and in doing so, they use a microscope to evaluate them rather than the FACS instrument.

Recently, researchers have applied a genetic engineering approach that uses fluorescence but is not dependent on cell surface markers. The importance of this new technique is that it allows the tracking of stem cells as they differentiate or become specialized. Scientists have inserted into a stem cell a "reporter gene" called green fluorescent protein. The gene is only activated or "reports" when cells are undifferentiated and is turned off when they become specialized. After activation, the gene directs the stem cells to produce a protein that fluoresces in a brilliant green color.

12.6.3 Future Perspectives in Myocardial Repair and Regeneration

The refinement of nuclear transfer, cybrid and cell fusion techniques may allow further engineering of stem cells to provide cardio protection or stimulate antioxidant

or antiapoptotic responses in the myocardium. These cell engineering techniques might also allow the specific targeting of mitochondrial-based cytopathies. To identify aspects of the cardiac milieu that may contribute to the growth and development of transplanted myoblasts *in vivo*, 3-dimensional matrices have been designed to serve as a novel *in vitro* system that mimic some aspects of the electrical and biochemical environment of the native myocardium. These structures may allow a finer resolution of electrical and biochemical signals that may be involved in myoblast proliferation and plasticity. Myoblasts have been grown on 3D polyglycolic acid mesh scaffolds under controlled conditions in the presence of cardiac-like electrical current fluxes and in the presence of culture medium that had been conditioned by mature cardiomyocytes. Such scaffolds containing either fetal or neonatal aggregates of contracting cardiac cells have been used to generate artificial cardiac grafts transplanted into injured myocardium with recuperation of ventricular function and formation of functional gap junctions between the grafted cells and the myocardium (Table 12.9).

The combination of gene therapy and stem cell engineering is an attractive approach for treating cardiac disorders. Overexpression (and in some cases, inhibition of expression) of specific proteins can result in striking changes in cardiomyocytes and in cardiac phenotype. Specific cardiomyocyte functions, including ion channel, cardiac conduction, contractility, and myocyte proliferation, have been shown to be effected by the gene transfer and expression of specific proteins. Cell-based therapies for injured or dysfunctional hearts can be enhanced by the use of *ex vivo* genetically modified stem cells to deliver genes and proteins. For instance, transplanted MSCs have been shown to be effective devices to deliver channel proteins involved in pacemaking activity (e.g., channel protein HCN2), resulting in the modification of cardiac rhythm *in vivo*.[35,126,141–143]

Several open questions are likely to be answered in the future:

1. What is the optimal time of delivery after acute myocardial infarction?
2. Is there a dose–response relationship?
3. How do different cell types compare?
4. The mechanism by which stem and progenitor cells achieve a functional improvement, which is difficult to test in the clinical scenario. In chronic ischemic HF, a superimposed question is whether identifying hibernating myocardium to direct cell therapy is essential to an effective outcome. The treatment for nonischemic heart disease is not yet addressed.

12.6.4 New Method Helps Stem Cells Find Damaged Tissue Better

Because the ability of stem cells migrating to damaged areas is well known, stem cells also have the ability to detect proteins that are secreted from the damaged tissue. Stem cells are chemotactic to detect movement (as amoeba, white blood cells attracted to chemicals and the movement around it). Research teams compared stem

TABLE 12.9 Myocardial Transplants: Advantages and Limitations Associated with Cell Type

Cell Type	Source	Advantages	Limitations
Cardiac stem cells	Allogenic fetal, neonatal, or adult heart	1. Recognition of myocardial growth factors and recruitment to myocardium are likely faster and more efficient than with other cell types 2. *In vivo* electrical coupling of transplanted cells to exiting myocardium has been demonstrated	1. Poor cell growth *in vitro* 2. Transplanted cells are very sensitive to ischemic insult and apoptotic cell death 3. Availability from fetal, neonatal, or adult sources is low at present; likely immune rejection; fetal and neonatal cells pose ethical difficulties
Skeletal myoblast	Autologous skeletal muscle biopsy	1. Cells proliferate *in vitro* (allowing for autologous transplant) 2. Ischemia resistant 3. Transplanted myoblasts can differentiate into slow-twitch myocytes (similar to cardiomyocytes), enabling cellular cardiomyoplasty 4. Reduces progressive ventricular dilatation and improves cardiac function 5. Can use adult cells	1. Likely do not develop new cardiomyocytes *in vivo* 2. Electrical coupling to surrounding myocardial cells is unclear (may cause arrhythmias) 3. Long-term stability of differentiated phenotype unknown
Adult bone marrow stem cells	Autologous bone marrow stromal cells (mesenchymal); bone marrow (endothelial progenitor cells)	1. Pluripotent stem cells can develop into cardiomyocytes 2. Stem cells are easy to isolate and grow well in culture 3. Neovascularization can occur at sue of myocardial scar reducing ischemia 4. Transdifferentiation of cells into cardiomyocyte *in vivo* has been shown 5. Can be derived from autologous source; no	1. New program of cell differentiation is required 2. Efficiency of the differentiation into adult cardiomyocytes appears limited 3. Signaling, stability and regulation of differentiation unknown

TABLE 12.9 (*Continued*)

Cell Type	Source	Advantages	Limitations
		immune-suppression treatment 6. Can improve myocardial contractile function	
Embryonic stem cells	Allogenic blastocyst (inner mass)	1. Easy propagation and well-defined cardiomyocyte differentiation process 2. *In vivo* electrical coupling of transplanted cells to existing myocardial cells 3. Pluripotent cells	1. Potential for tumor formation and immune rejection (allogenic) 2. Incomplete response to physiological stimuli 3. Legal and ethical issues 4. Donor availability

Source: Adapted from Ref. [17].

cell activity in the environment of chemokines and growth factors. Both factors induce the migration of stem cells; however, growth hormone appeared to be more effective. In particular, PDGF-AB, TGF-β1, TNF-α were exposed to growth hormones to observe the active migration of stem cells.

Interestingly, the factors that enhance the migration of stem cells exhibited improvement when used to stimulate stem cells. Among these factors, TNF-α showed the best response from stem cells. It was confirmed that stem cells' homing effect improved up to 4.4 times when stimulated by chemotactic chemokines and growth hormones.

12.6.5 Shortcomings in Stem Cell Applications

Use of ESCs for research involves the destruction of blastocysts formed from laboratory-fertilized human eggs. For those who believe that life begins at conception, the blastocyst is a human life, and to destroy it is unacceptable and immoral.

The range of different types of cells that stem cells can change into may be limited to a set of cells that may not be useful for certain diseases. The complexity of individual diseases will govern whether stem cell therapy is applicable.

Another possible restriction is the problem of tissue rejection when stem cells from other sources are used to treat a person who is tissue incompatible. This is a situation in which adult stem cells from the patient could have great use because they would not be recognized as foreign by their body.

Use of stem cell lines from alternative nonembryonic sources has received more attention in recent years and has already been demonstrated as a successful option for treatment of certain diseases. For example, adult stem cells can be used to replace blood cell–forming cells killed during chemotherapy in bone marrow transplant patients.

Biotech companies are researching techniques for cellular reprogramming of adult cells, use of amnionic fluid, or stem cell extraction techniques that do not damage the embryo and that provide alternatives for obtaining viable stem cell lines. ESCs can be isolated in greater numbers and are less limited in the number of cell types they can generate, which makes them attractive for study, but their current use has funding and ethical constraints. So the potential application of stem cell therapy to disease treatment is broad but not without its limits.

12.6.5.1 What Does the Future Hold? The potential for stem cell therapy is immense, and much of this potential is applicable to the use of adult stem cells, which makes things less complicated from the ethics point of view. However, with efforts from individual organizations and states underway to fund human ESC research, this field will also move forward rapidly. We think in the next 15 years that amazing things will be achieved in the field as research moves along at a quickening pace. Thus the future is bright for the field of stem cell research, its application to the treatment of a variety of diseases, and the resulting enhancement to the quality of life for many.

On March 9, 2009, President Barack Obama removed certain restrictions on federal funding for research involving new lines of human embryonic stem cells. Federal funding originating from current appropriations to the Department of Health and Human Services (including the National Institutes of Health) under the Omnibus Appropriations Act of 2009, remains prohibited under the Dickey Amendment for (1) the creation of a human embryo for research purposes; or (2) research in which a human embryo or embryos are destroyed, discarded, or knowingly subjected to risk of injury or death greater than that allowed for research on fetuses in utero.

12.6.6 Stem Cell Research Controversy

Stem cell research is governed by country-specific guidelines and involves ethical concerns and legal and religious issues.

12.6.7 Problems with Embryonic Stem Cell Research

12.6.7.1 The Issue of Who or What Cloning technology destroys the scientific and legal basis of distinguishing a preembryo from an embryo, the popular distinction made at 14 days after conception. This is significant because this distinction determines the handling and treatment of human life less than 14 days old, which is so basic to all ESC research. There is no real preembryo–embryo distinction, and that all human life begins at conception. Therefore, as a nation, we should rightly adjust the moral and legal treatment and status of all embryos to people, not property, from the point of conception.

12.6.7.2 Embryonic Stem Cell Research is Related to Human Cloning Understanding how ESC research and human cloning relate requires delineation between the two forms of human cloning: reproductive and therapeutic. Reproductive cloning creates a later-born twin from a single cell of another person by transplanting the DNA

of the adult cell into a human egg whose nucleus has been removed. This process is somatic cell nuclear transfer. In this procedure, the resulting embryo is implanted in a woman and carried to birth. Therapeutic cloning begins with the same procedure as reproductive cloning. Whereas the goal of reproductive cloning is to produce a baby, the goal of therapeutic cloning is to produce ESCs for research or treatment.

12.6.7.3 There is Law that Could Apply to ESCR Originally attached to the 1995 Health and Human Services (HHS) appropriations bill, the "Dickey Amendment" has prohibited federal funding of "any research in which a human embryo or embryos are destroyed, discarded or knowingly subjected to risk of injury or death." Unfortunately, there are no laws to protect preembryos (embryos younger than 14 days old) or that prohibit private individuals, research firms, or pharmaceutical companies from forming, manipulating, or destroying stem cells, human clones, or embryos.

12.6.7.4 ESCR Currently has Major Disadvantages One minor complication is that use of human ESCs requires lifelong use of drugs to prevent rejection of the tissue. Another more serious disadvantage is that using ESCs can produce tumors from rapid growth when injected into adult patients. A third disadvantage reported in the March 8, 2001, *New England Journal of Medicine* was of tragic side effects from an experiment involving the insertion of fetal brain cells into the brains of patients with Parkinson's disease. Results included uncontrollable movements, including writhing, twisting, head jerking, arm flailing, and constant chewing. Fourth, a recent report in the *Journal Science* reported that mice cloned from ESCs were genetically defective. Finally, the research may be hampered because many of the existing stem cell lines were grown with the necessary help of mouse cells. If any of this research is to turn into treatments, it will need approval from the FDA, which requires special safeguards to prevent transmission of animal diseases to people. It is unclear how many of these cell lines were developed with the safeguards in place. This leads to a host of problems related to transgenic issues.

12.6.8 Challenges Remain for Stem Cell Therapies

There are many challenges to making stem cell therapies, such as regenerative medicine, actually work in a therapeutic setting. We might be able to harvest stem cells, from either blastocysts or by creating pluripotent cells from already differentiated tissues, but that is really only the beginning of a medically viable process. After a cell line is cultured in a maintainable way, the following questions remain:

- How to direct differentiation into the desired tissue type and optimizing growth conditions and the physical environment for cell cultures or for growing organs for transplantation
- How to inject and transport stem cells to the target location in the body
- Finding ways to generate induced pluripotent stem cells without inducing tumor formation in future recipients of stem cell therapies.

Reprogramming of human somatic cells uses readily accessible tissue, such as skin or blood, to generate embryonic-like iPSCs. This procedure has been applied to somatic cells from patients who are classified into a disease group, thus creating "disease-specific" iPSCs. Here, the challenges and assumptions are in creating a disease model from a single cell of the patient. Both the kinetics of disease onset and progression as well as the spatial localization of disease in the patient's body are challenges to disease modeling. New tools in genetic modification, reprogramming, biomaterials, and animal models can be used for addressing these challenges.

Despite these many hurdles and the newness of the technology, there are already some glimmers of hope for clinical applications of hiPSCs. Diseases of the retina may offer an early test bed for hiPSC-derived cells in the form of retinal pigmented epithelium, given the relative isolation of the tissue and the small number of cells required. A second intriguing possibility would be the use of hiPSCs to produce functional cells for use in extracorporeal applications, such as mature hepatocytes for use in bioartificial livers. If such early applications prove successful, it may help to allay concerns over safety and increase public and regulatory acceptance of the clinical use of hiPSCs, enabling them to establish a solid footing before attempts are made at treating more complex and deeply rooted disorders. At the same time, it will be important for cell therapy pioneers to investigate alternative routes for funding their work, so as to ensure the standards of safety and efficacy expected of small molecules are also met by cellular applications.

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