16.1 BACKGROUND

Historically, biomaterial implants have been designed to replace a specific function, usually mechanical, and were considered ideal if they elicited little or no response in vivo. For instance, synthetic vascular grafts have typically been made of inert materials such as expanded polytetrafluoroethylene (Teflon) with the necessary mechanical strength to support the forces created by pulsatile blood flow. Likewise, materials for orthopedic screws and plates for bone fixation were usually metals, chosen for their mechanical strength. However, synthetic small-diameter vascular grafts fail by thrombosis, and orthopedic implants can weaken existing bone due to stress shielding or fail due to implant loosening. In addition, most surface-modification approaches to improve integration of the implant with the host tissue have not been very successful over the last 20 years. These failures demonstrate that synthetic materials alone cannot fully replace all the functions (structural, mechanical, biochemical, metabolic, etc.) that the original tissue provided.

Due to new understanding from a molecular biology perspective, it is clear that most tissue has two categories of components: (1) structural and (2) biochemical. The field of tissue engineering considers both these components in the design of implants to promote regeneration and facilitate better integration of implants. With a growth rate of 22.5 percent per annum (based on the annual spending in 1994 and 1997), tissue engineering is a rapidly expanding field. In 1997, over $450 million was allocated for tissue engineering to fund the research efforts of nearly 2500 scientists and support staff (Lysaght et al., 1998). Tissue-engineered skin products (Apligraf from Organogenesis, Transcyte from Advanced Tissue Sciences) and bioengineered cartilage (Carticel from Genzyme Tissue Repair) are examples of commercially available treatments, and many other tissue regeneration products are currently in clinical trials.

Using the tissue-engineering strategy, materials designed for tissue regeneration attempt to control the physiological response to the implanted biomaterial by mimicking the two-component structure of natural tissue. A variety of techniques have been implemented in this approach to the design of regenerative biomaterials, as listed in Table 16.1. Tissue engineers typically use synthetic or natural
biomaterials to achieve the structural design parameters. The design space for optimizing the structural parameters includes the physical, mechanical, and chemical characteristics of the material. The biochemical parameters are incorporated via immobilized signals, diffusible chemoattractive agents, or living cells. The critical factors that promote tissue growth in vivo can be determined by examining environments that stimulate tissue generation and regeneration in the body, such as those found during development, tissue remodeling, and wound healing. By providing similar cues, regenerative materials can function in a supportive role, aiding the biological processes when necessary, rather than permanently replacing a lost function. The materials discussed in this chapter are listed in Table 16.2.

### 16.2 STRUCTURAL COMPONENT

In order to properly guide tissue regeneration, a biomaterial ought to satisfy the structural requirements of the native tissue. For each type of tissue and application, the implant must have the correct physical architecture with the appropriate mechanical and chemical properties. Table 16.3 provides examples of approaches that utilize these properties for tissue regeneration.

#### 16.2.1 Physical Properties

In tissue regeneration, both natural and synthetic materials contribute to the spectrum of physical properties displayed in biomaterials today. Collagen, a naturally occurring bioresorbable protein, is commonly used as a scaffold for tissue regeneration. The most frequently used collagen is type I collagen, but other types may also be appropriate. However, natural materials such as collagen can only provide a limited range of properties, and they may be immunogenic (Madsen and Mooney, 2000). Synthetic materials, including the biodegradable aliphatic polyesters, namely, poly(L-lactic acid) (PLLA), poly(D,L-lactic acid) (PDLLA), poly(glycolic acid) (PGA), and poly(lactide-co-glycolide) copolymers (PLGA), can be formed to provide a range of physical properties and may therefore offer greater flexibility. These materials are often produced in a less expensive, more
reproducible manner relative to natural materials. However, processing often involves the use of high temperatures or harsh organic solvents that may preclude the incorporation of sensitive proteins or nucleic acids. Also, residual solvent in the final product may detrimentally affect the in vivo response to the material. Consequently, while evaluating the physical properties of an implant, including shape, size, porosity, three-dimensionality, and surface topography, other innate characteristics of the material should also be considered.

**Shape.** The overall shape of a biomaterial implant is often dictated by the application. PLGA polymers (Giordano et al., 1997; Lu et al., 1998; Thomson et al., 1996) and collagen (Tiller et al., 2001) have been formed into thin films to facilitate the growth of cell monolayers, including epithelium. For perivascular treatment of intimal hyperplasia in blood vessels, alginate hydrogels containing PLGA drug-delivery systems were fabricated into perivascular wraps (Edelman et al., 2000). In the nervous system, polymeric degradable rods made of PDLLA foams mixed with poly(ethylene oxide)-block-poly(D,L-lactide) (PELA) have been used for spinal cord regeneration in rats (Maquet et al., 2001), and porous conduits of PLGA, PLLA (Widmer et al., 1998), or poly(L-lactide-co-6-caprolactone) (Giardino et al., 1999) have been fabricated to facilitate regeneration of peripheral nerves (Evans et al., 1999a). To form tubular shapes for applications such as intestinal tissue engineering, PLGA copolymers have been made into porous films, formed into tubular structures, and implanted in vivo to develop tubular fibrovascular tissue (Mooney et al., 1994). Tissues with complex three-dimensional (3D) shapes require a more sophisticated technique to fabricate appropriately shaped implants. One study produced a dome-shaped bone formation in a rabbit calvarial defect using PLLA-tricalcium phosphate (TCP) matrices molded into a dome-shaped structure (Lee et al., 2001). For joint repair, the specific 3D shapes of the metacarpal-phalangeal pieces have been replicated with PLLA and PGLA using a lamination technique to precisely control the profile (Mikos et al., 1993). These studies illustrate the variety of methods available to create almost any desired shape.
In addition to large, continuous shapes, smaller, discontinuous materials have also been used for tissue regeneration, either for delivery of growth factors or as a scaffold to culture and transport cells to diseased or injured areas. To act as a drug-delivery system, microparticles made of PLGA and poly(ethylene glycol) (PEG) were loaded with transforming growth factor-β (TGF-β). The loaded microparticles increased the proliferation of rat marrow stromal cells (Peter et al., 2000). When designed as cell scaffolds, microcarriers made of collagen have been used to culture human chondrocytes (Frondoza et al., 1996), and fibrin microbeads have been used to transport fibroblasts for skin wound-healing applications (Gorodetsky et al., 1999). Glass and collagen-coated dextran microspheres have been used to transplant cells for neuroregeneration (Borlongan et al., 1998; Saporta et al., 1997). Also for neuroregeneration, PC 12 cells have been microencapsulated in 75:25 hydroxyethyl methacrylate-methyl methacrylate copolymers (Vallbacka et al., 2001), as well as in alginate-polylysine-alginate (Winn et al., 1991). Small microparticles are advantageous because, unlike large implants, suspensions of microparticles are usually injectable.

**Size.** The overall size of an implant is important, especially with respect to the diffusion of oxygen and nutrients within the implant. It is essential for regenerating tissue to have access to the appropriate nutrients as well as a method of waste removal, and the oxygen supply is often the limiting factor. Cells typically require an oxygen partial pressure \( P_{O_2} \) of 1 to 3 mmHg for basic cellular metabolism (Hunter et al., 1999). Due to the capillary \( P_{O_2} \) and the oxygen diffusion coefficient in tissue, cells are usually found no more than 50 µm from a capillary in vivo (Guyton and Hall, 1996b). When biomaterial scaffolds have a thickness significantly greater than 100 µm, oxygen deficits may occur, depending on the diffusive properties of the scaffold. When PLLA sponges infiltrated with polyvinyl
alcohol (PVA) were formed into 1-mm-thick samples for the seeding and delivery of hepatocytes, the cells on the interior of the sponges necrosed in vivo (Mooney et al., 1995), probably because the oxygen and nutrient demands of the cells exceeded the supply. Another study seeded smooth muscle cells (SMCs) onto PGA fibers for transplantation into rats. After 18 days, viable SMCs were found at the edges of the implant, but cells more than 100 \(\mu\text{m}\) into the implant did not survive (Eiselt et al., 1998). Consequently, if a cellular implant is sufficiently large, it will require enhanced oxygen transport, perhaps via an internal blood supply, to support the cells deep within the implant.

Blood vessels naturally invade foreign materials as part of the wound-healing response, so macroporous scaffolds will eventually vascularize. However, this process is lengthy, taking over 3 weeks even for relatively small (5 mm) PLLA scaffolds (Voigt et al., 1999). To avoid the development of a necrotic core, implant size should be based on the diffusive properties of the material as well as the metabolic needs of the cells. Multicell spheroids cultured in soft agar enlarged until they reached a critical size, after which they ceased to expand. Although the critical size ranged from 2.4 to 4.0 mm, the first cells to necrose were greater than 150 to 200 \(\mu\text{m}\) from the surface, probably due to the limitation of oxygen diffusion (Folkman and Hochberg, 1973). In this study, the allowable distance between the viable cells within the spheroids and the nutrient/oxygen source was larger than the typical in vivo distance between cells and blood vessels (50 \(\mu\text{m}\)), most likely due to the diffusive properties of the agar and the density and metabolism of the cells. Nevertheless, diffusion alone was not sufficient to sustain cells deep within the spheroids. To overcome limitations such as this, a biochemical stimulus to enhance nutrient influx can be included in the material, as discussed later.

Porosity. A biomaterial for tissue regeneration must be porous enough to allow cells to invade the material, with ample surface area for an appropriate quantity of cells to adhere and proliferate. Studies have shown that creating pores in polycarbonate and polyester membranes enhanced corneal epithelial tissue migration (Steele et al., 2000), and the introduction of pores into PLGA foams increased rat hepatocyte adhesion (Ranucci and Moghe, 1999). The surface area for cell attachment is correlated with the porosity of the polymer, so highly porous PLGA foams have the advantage of increased available surface area to support cellular attachment and growth (Zheng et al., 1998). The porosity of a biomaterial also affects the diffusion of oxygen and nutrients within the scaffold, as well as the rate of neovascularization (Eiselt et al., 1998). Larger pores in the biomaterial may allow for improved mass transport and neovascularization (Zhang and Ma, 2000). In addition, pore shape can be adjusted to enhance tissue regeneration. Biodegradable PDLLA-PELA foams have been made with longitudinally oriented pores to facilitate spinal cord regeneration (Maquet et al., 2001).

Often, a specific pore size enhances cellular activity, and the optimal pore diameter depends on the cell type. In one set of studies, the nominal pore size of porous polycarbonate membranes affected epithelial tissue migration (Steele et al., 2000) and the formation of a continuous basement membrane (Evans et al., 1999b). Likewise, when the average pore radius of agarose hydrogels (Fig. 16.1) was below a critical threshold, neurite extension from rat embryonic striatal cells and embryonic chick dorsal root ganglia (DRGs) was inhibited (Bellamkonda et al., 1995; Dillon et al., 1998). Also, cellulose membranes with 0.2-\(\mu\text{m}\) pores were unable to support cell ingrowth, but when the pore size was increased to 8 \(\mu\text{m}\), cells were able to invade the membranes (Padera and Colton, 1996). Interestingly, rat hepatocyte attachment to 3D PLGA foams of varying porosity showed no preference for any given porosity. However, when protein secretion was evaluated, cells cultured on foams with supracellular-sized (67 \(\mu\text{m}\)) pores had a significantly increased secretion of albumin as compared with cells on foams with intermediate-sized (17 \(\mu\text{m}\)) or subcellular-sized (3 \(\mu\text{m}\)) pores. This effect may have been due to increased cell-cell contacts in the scaffolds with supracellular-sized pores (Ranucci and Moghe, 1999). These results indicate that while pore size and shape should be optimized for each application, it may not be sufficient to use only one fundamental parameter, such as cell attachment, to determine the best pore morphology.

Since the optimal pore size depends on the application, it is important to have control over the porosity of materials. Novel methods of fabricating a porous matrix include using a 3D paraffin mold to create a spherical pore network (Ma and Choi, 2001); using sugar particles, disks, and fibers to produce a variety of controlled porous PLLA structures (Zhang and Ma, 2000); and using highpressure gas
foaming in combination with particulate leaching to create porous scaffolds from PLGA copolymers without the use of organic solvents (Murphy and Mooney, 1999). The porosity of PLGA-PEG blends, fabricated by solvent casting and particulate leaching methods, was significantly dependent on the initial salt fraction as well as the ratio of PLGA to PEG (Wake et al., 1996). Similarly, the porosity of collagen-chitosan matrices was controlled by changing the component ratios, with increased amounts of chitosan resulting in smaller pores. As the chitosan concentration increased, proliferation of a human hemopoietic cell line decreased, perhaps due to the smaller pore size. Notably, cell viability remained unchanged (Tan et al., 2001). These collagen-chitosan matrices may be suitable for applications such as cell encapsulation, where it is desirable for cells to remain viable but not highly proliferative to avoid overcrowding the scaffold. Even with a thorough, controlled design of initial porosity as described in these studies, processes including fibrous encapsulation, scaffold degradation, cellular ingrowth, and extracellular matrix (ECM) production must be considered because they may alter the scaffold porosity after implantation.

Three-Dimensionality. Since tissues in vivo are generally 3D constructs, cells cultured in 3D substrates are more likely to reflect in vivo scenarios. Culturing cells in vitro on two-dimensional (2D) substrates rather than in three dimensions has been shown to affect cell phenotype. Hepatocytes cultured in alginate beads (400 µm diameter) secreted more albumin, fibrinogen, α1-antitrypsin, α1-acid glycoprotein, and prothrombin than a similar cell density in a 2D monolayer culture. Moreover, the levels produced by the cells on the alginate beads approached those of normal hepatocytes in vivo, perhaps because the 3D cell architecture mimicked that found in vivo (Selden et al., 1999). Primary human chondrocytes cultured on 2D substrates transitioned to a fibroblastoid cell phenotype. When cultured on 3D collagen microcarriers, they reverted back to the original chondrocytic phenotype (Frondoza et al., 1996). Similarly, when neurons were cultured on 2D agarose cultures, they did not adhere and grow. However, when embedded in 3D agarose hydrogels with the identical chemistry, the neurons were viable and extended long processes (Bellamkonda et al., unpublished observations). Photomicrographs of growth cones extending from primary neurons illustrate the difference between the growth-cone morphology on 2D tissue culture substrates and the 3D
morphology in agarose hydrogel (Fig. 16.2). These examples show that cell phenotype and morphology depend on the dimensionality of the culture conditions.

Despite this fact, many in vitro studies related to cell migration and proliferation have been performed on 2D tissue culture substrates due to the relative simplicity of these experiments. Some groups have performed transmigration assays (Gosiewska et al., 2001) and proliferation assays (Chu et al., 1995; Tan et al., 2001) with 3D cultures. However, 3D analysis of cultures can be difficult because cells cannot be manipulated or imaged as easily as those on 2D substrates. Live imaging of cells cultured in 3D scaffolds via light microscopy is challenging due to the thickness and nontransparent nature of the 3D material.

FIGURE 16.2 Images of growth cones (arrows) extending from embryonic chick dorsal root ganglia in vitro: (A) growth cone morphology on 2D substrates is typically flat and extended (image captured via light microscopy); (B) in 3D agarose hydrogel cultures, growth cones exhibit a 3D ellipsoidal shape (image is one optical slice through a 3D growth cone captured via confocal microscopy) (bar = 25 μm).
In addition, techniques such as immunostaining require adaptation for 3D cultures in vitro. Often, when in vitro experiments are performed using 3D scaffolds, the cultures are either fixed and sectioned prior to staining (Gosiewska et al., 2001; Schreiber et al., 1999) or else stained in situ and then sectioned for analysis (Attawia et al., 1995; Chu et al., 1995; Holy et al., 2000). Recently, a method for staining PLGA scaffolds in situ and then performing a histological analysis has been published (Holy and Yakubovich, 2000). Immunostaining neurites and growth cones is not as straightforward. Three dimensional cultures of primary neurons in agarose hydrogels can be imaged via light microscopy because the hydrogels are transparent and the cell density is low (Dillon et al., 2000). Nevertheless, staining and sectioning the cultures are not recommended because the fine neuronal processes may be destroyed. To maintain the integrity of the neuronal processes, a modified staining method is under development in our laboratory. This method involves partially digesting the hydrogel to increase the porosity and allow the unimpeded diffusion of antibodies and fluorophores in and out of the hydrogel. While culturing in 3D substrates can be useful in maintaining normal phenotypes, significant challenges with cultures, qualitative visualization, and assays remain.

**Surface Topography.** Modifying the surface topography and surface roughness of a material is another approach to enhancing interactions between the implant and the tissue. When the topography of the subendothelial extracellular matrix was replicated with biomedical polyurethane, bovine aortic endothelial cells (BAECs) spread more rapidly and appeared more like cells in a native environment as compared with cells on nontextured polyurethane (Goodman et al., 1996). To determine the effect of surface roughness on bone augmentation, smooth and grit-blasted (textured) titanium cylinders were implanted in rabbits. Although both types of surfaces resulted in similar amounts of trabecular bone formation, the grit-blasted titanium had a larger area of bone directly contacting the implant (Lundgren et al., 1999). As these studies illustrate, cells sometimes prefer a textured surface to a smooth surface, perhaps due to the increased surface area available for cell attachment and tissue integration.

Grooves have also been used to adjust the surface topography of materials. By creating microgrooves on tissue culture polystyrene, a significantly higher number of rat dermal fibroblasts attached onto and aligned actin filaments with 1-µm-wide grooves relative to smooth surfaces and surfaces with grooves greater than 2 µm wide (Walboomers et al., 2000). Similarly, chick embryo cerebral neurons and their processes have been shown to follow micron-but not nanometer-scale grooves faithfully in vitro (Clark et al., 1990, 1991). *Xenopus* spinal cord neurons cultured on grooved quartz extended neurites parallel to the grooves, regardless of groove dimensions, but rat hippocampal neurons extended neurites parallel to deep, wide grooves and perpendicular to shallow, narrow grooves (Rajnicek et al., 1997). Also, when polymeric guidance channels were used for rat sciatic nerve regeneration, the topography of the luminal surface affected the regeneration (Guenard et al., 1991). Only channels with a smooth inner surface produced a discrete nerve cable with microfascicles consisting of myelinated axons (Aebischer et al., 1990). Due to the critical influence of surface topography on tissue organization and response, topography should be considered when designing regenerative biomaterials.

### 16.2.2 Mechanical Properties

Biomaterials to regenerate load-bearing tissues, including bone, cartilage, and blood vessels, have obvious requirements for mechanical properties such as modulus of elasticity, tensile and shear strength, and compliance. For instance, biodegradable bone cement designed to aid in the repair of broken bones or to fill bone voids must have mechanical properties similar to those of the native bone until the bone heals (Peter et al., 1998a). For such implants, composite reinforced materials may be necessary to achieve the desired mechanical properties. Moreover, in the regeneration of soft tissue such as cartilage, the development of the appropriate mechanical properties may be critically dependent on the mechanical conditioning imposed in vitro.

Mechanical properties are also important for other applications where mechanical strength is not the primary function. One such application is electrodes for stimulation or recording in the brain. Silicon microelectrodes inevitably seem to elicit astroglial scar due to mechanical mismatch with the
host neural tissue and lack of integration between the implant and the brain tissue (Maynard et al., 2000), and this fibrous scarring can lead to electrode failure (Williams et al., 1999). It has also been directly demonstrated that the rate of neurite extension is affected by the stiffness of the material in which the neuron is growing (Balgude et al., 2001). Soft tissues like the nervous system should not be overlooked when considering mechanical properties.

**Reinforced Composites.** Bone regeneration and fixation require strong materials, so applicable biomaterials must have the strength to sustain the mechanical loads experienced in bone. However, many materials with desirable physical and chemical properties, such as biodegradable polymers, are often unable to provide this strength by themselves. By combining two different materials, reinforced composites with the joint properties of both materials can be fabricated.

PLLA, PGA, and PLGA are common scaffolds for a number of tissue-regeneration applications, including bone regeneration. The Bioscrew, a PLLA screw, has been found to perform comparably with metal screws as a fixation device for patellar tendon autografts. Moreover, the Bioscrew is completely degradable and has the ability to compress slightly and conform to its surroundings when inserted into bone (Barber et al., 2000). However, most forms of PLLA, PDLLA, PGA, and PLGA are not high-strength materials, and adjusting physical parameters to enhance tissue ingrowth, such as by increasing the pore size, leads to a further decrease in the mechanical strength (Ma and Choi, 2001). Reinforcing techniques via the addition of mineral and ceramic components have been developed to strengthen these and other materials for load-bearing applications.

Hydroxyapatite (HA), a mineral component found in bone, is commonly used to reinforce polymers for bone regeneration because it can enhance mechanical properties as well as provide osteoinductive properties. Absorbable high-strength composites of PLLA reinforced with HA are being considered for fixation devices in bone. These composite rods have an initial bending strength exceeding that of human cortical bone and have promoted greater bone contact and better bone integration than PLLA without HA in a rabbit bone defect model (Furukawa et al., 2000). PLGA has also been strengthened with HA to form PLGA-HA composites with an elastic modulus and yield strength similar to those of cancellous bone (Devin et al., 1996). Short HA fibers have been used to reinforce PLGA and produce stronger composite foams for bone regeneration (Thomson et al., 1998). Based on these and other studies, it can be concluded that HA incorporated into biodegradable polymers can increase the strength of the composite to a level useful for orthopedic implants.

The bioactive ceramic β-tricalcium phosphate (β-TCP) has also been used to develop composite materials for bone regeneration. Poly(propylene fumarate) (PPF) has been strengthened by incorporating β-TCP to result in a biodegradable composite material with sufficient mechanical strength to temporarily replace trabecular bone (Yaszemski et al., 1996). It is notable that the mechanical properties of the composite actually increased during degradation, maintaining a compressive strength of at least 5 MPa and a compressive modulus greater than 50 MPa for 3 (Peter et al., 1998b) to 12 (Yaszemski et al., 1996) weeks depending on the composition. Materials that maintain their mechanical strength in this manner are useful for tissue regeneration because they allow a gradual, smoother replacement of the temporary biomaterial with the host matrix and cells. If the mechanical properties of a biomaterial decrease very rapidly, it may be beneficial to overengineer the initial implant to compensate for the loss.

Reinforcements are used for nonpolymeric materials as well. HA coatings on titanium bone fixtures increased contact between the native bone and the implant and provided greater shear and antitorque strength (Meffert, 1999). Microporous calcium phosphate ceramics (75 percent HA and 25 percent β-TCP) generally have weak mechanical properties under compression. When the pores were filled with calcium phosphate cement consisting of β-TCP and dicalcium phosphate dihydrate, the mechanical strength of the ceramic improved without compromising the bone healing and regeneration process (Frayssinet et al., 2000). By reinforcing available materials with components that provide strength as well as osteoinductive properties, bone regeneration can be enhanced.

**Mechanical Conditioning.** Tissue regeneration has been shown to benefit from external mechanical stimuli. One study demonstrated that constant mechanical tension elongated the axon bundles of synapsed primary embryonic rat cortical neurons in vitro (Smith et al., 2001). Moreover, certain
tissues require mechanical loads for the generation of proper cell phenotypes. When in vitro culture conditions emulate in vivo loading, native cell phenotypes can be maintained.

Blood vessels in vivo are mechanically loaded by the pulsatile flow of the bloodstream. To simulate these conditions, tissue-engineered blood vessels are often grown under pulsatile flow conditions. Tissue-engineered arteries grown in vitro under pulsatile flow appeared more similar to native arteries than vessels not cultured under flow (Niklason et al., 1999), and cell-seeded vascular grafts cultured under pulsatile flow demonstrated superior mechanical strength relative to static control grafts (Hoerstrup et al., 2001). Thus in vitro cultures of blood vessels benefit from mechanical conditioning similar to the in vivo mechanical loading.

Cartilage is another tissue that experiences mechanical loads in vivo. Explants of healthy cartilage respond to compressive loads and in particular to the release of those loads. The release of a static compressive load stimulated the chondrocytes, and continuous dynamic loading was able to stimulate or inhibit the biosynthesis of proteins depending on the amplitude and frequency of the compression (Sah et al., 1989; Steinmeyer and Knue, 1997). This behavior of native cartilage extends to chondrocytes cultured in vitro. Chondrocytes cultured on agarose disks were evaluated under static and dynamic compression. Dynamic compression resulted in an increase in proteoglycan and protein synthesis with time, whereas little change in synthesis was seen with static compression (Buschmann et al., 1995; Lee and Bader, 1997). Likewise, cyclic loading was able to stimulate chick mesenchymal stem cells cultured on agarose hydrogels to differentiate into chondrocytes, making them useful for cartilage regeneration applications (Elder et al., 2000). Moreover, the chondrogenesis depended on the frequency and duration of the cyclic compressive load, indicating the similarity between these constructs and native cartilage (Elder et al., 2001). These and other studies have established the importance of mechanical stimulation for chondrocytes and offer a possible approach to enhance cartilage generation in vitro and regeneration in vivo.

**Electrical Stimuli.** Electrically active materials have also been used to encourage tissue growth. The use of piezoelectric materials made of vinylidene fluoride–trifluoroethylene copolymer [P(VDFTrFE)] enhanced peripheral nerve regeneration in vivo (Fine et al., 1991), and when PC 12 cells were cultured on oxidized polypyrrole, the application of an electrical stimulus resulted in enhanced neurite extension (Schmidt et al., 1997), as shown in Fig. 16.3. Implant vascularization was enhanced when bilayer films of polypyrrole–hyaluronic acid and polypyrrole-poly(styrenesulfonate) were implanted subcutaneously (Collier et al., 2000). These types of electrical stimuli can be used in conjunction with a biomaterial to promote tissue regrowth.

### 16.2.3 Chemical Properties

The chemistry of a material affects the interactions that occur between the implant and its surrounding environment, including protein adsorption, cellular response, and bioresorption. In particular, the hydrophilicity, charge, and degradability of the material can impact tissue regeneration.

**Hydrophilicity.** One aspect of material chemistry that affects cell behavior is the hydrophilicity of the biomaterial. Most mammalian cells are anchorage-dependent and only viable when attached to a substrate in a receptor-mediated fashion. Since they have no receptors for most synthetic biomaterials, cells will not attach to bare materials in a receptor-mediated manner. However, protein adsorption onto biomaterials results in a permissible surface for cell attachment. It has been shown that moderately hydrophilic surfaces, in the presence of serum proteins, often support greater cell attachment (Chang et al., 1999; Khang et al., 1999; van Wachem et al., 1985), spreading (Webb et al., 1998), and normal phenotypes (McClary et al., 2000) relative to hydrophobic or highly hydrophilic surfaces. Focal contacts and stress fibers in cells on this moderately hydrophilic surfaces are well defined, indicating active binding and outside-in signaling due to the proteins adsorbed onto the surface (McClary et al., 2000). The increased cell attachment on moderately hydrophilic surfaces could be due to preferential binding of cells to ECM proteins that adsorbed to the surfaces (Khang et al., 1999).
Spatial control of cells is achieved by including zones that permit cell attachment with adjoining zones that inhibit attachment. Very hydrophilic surfaces are relatively resistant to protein adsorption and can be used as an inhibitory surface to generate patterns of attached cells on a biomaterial. High-molecular-weight PEG (above 18,500 g/mol) is known to be resistant to protein adsorption and cell adhesion and can be modified for the covalent coupling of other molecules (Desai and Hubbell, 1991; Gombotz et al, 1991). When PEG chains were grafted onto silica films, significantly less protein adsorption was observed as compared with unmodified surfaces (Alcantar et al., 2000). PEG chains can also reduce cell adhesion. Collagen, known for its ability to promote cell attachment, can be modified into a nonpermissive substrate by attaching PEG to the collagen (PEG-collagen) (Tiller et al., 2001). These and other methods can be used to create regions of differing hydrophilicity to control cell micropatterns on surfaces.

![Figure 16.3 PC 12 cell differentiation on polypyrrole (PP) without (A) and with (B) application of an electric potential. PC 12 cells were grown on PP for 24 h in the presence of nerve growth factor and then exposed to electrical stimulation (100 mV) across the polymer film (B). Images were acquired 24 h after stimulation. Cells grown for 48 h but not subjected to electrical stimulation are shown for comparison (A) (bar = 100 µm). (Courtesy of C. Schmidt, Ph.D.)](image-url)
**Charge.** In addition to preferring moderately hydrophilic surfaces, cell attachment is improved on positively charged surfaces. Polymers that carry a positive charge at physiological pH augment cell attachment and growth in the presence of proteins (McKeehan and Ham, 1976). In the absence of proteins, cells still prefer positively charged hydrophilic surfaces to those with a neutral or negative charge (Webb et al., 1998). When polyllysine (a positively charged protein) was coated onto PLLA and PGLA nonwoven structures, chondrocyte attachment increased (Sittinger et al., 1996). When fluorinated ethylene propylene films were surface modified with patterns of amine groups, preadsorption with albumin resulted in neuroblastoma cell attachment along the amine patterns (Ranieri et al., 1993). Also, neurite extension, an important element of nerve tissue regeneration, has been shown to depend on the polarity of the substrate in which the cells are cultured. When embryonic chick DRGs were cultured in 3D agarose hydrogels, negatively charged dermatan sulfate coupled to the hydrogel inhibited neurite extension, whereas positively charged chitosan coupled the hydrogel enhanced neurite extension (Dillon et al., 2000). To determine the effect of charge on the ability of collagen to support cells, collagen was modified to have either a net positive or negative charge. However, the charge difference had minimal effect, with 90 percent of mouse fibroblasts and endothelial cells attaching after 60 minutes, regardless of the substrate’s net charge. When PEG-collagen, which did not support cell attachment, was given a net positive charge through amination, fibroblast and endothelial cell attachment returned to the levels of attachment on unmodified collagen (Tiller et al., 2001). As demonstrated by these studies, positive charges added to biomaterial scaffolds can induce tissue growth.

**Biodegradation.** Biomaterials for some applications such as total-hip replacements and synthetic vascular grafts are designed to be long-lived and even permanent, if possible. However, many tissue-regeneration implants are best designed using materials that can completely degrade in vivo to be replaced with autologous tissue. The lack of a synthetic remnant is advantageous because it reduces the likelihood of infection and chronic inflammation that are often seen with permanent implants. Biodegradable materials are also ideal for tissues that are still growing and developing, such as bones and heart valves in pediatric patients.

The chemistry of certain materials allows them to degrade in vivo, typically due to cleavable bonds. The most commonly used degradable polyester polymers include PLLA, PDLLA, PGA, and PLGA. These polymers undergo bulk degradation into lactic and glycolic acid by hydrolysis of the ester bonds. The degradation by-products are nontoxic and easily metabolized, and the rate of degradation can be controlled by the ratio of PLLA to PGA. Polyanhydrides are another type of biodegradable polymer used for drug delivery and tissue engineering. Due to the hydrophobicity of the polymer chains, they degrade by predictable surface erosion rather than bulk degradation, as observed with the polyesters. A variety of other synthetic and natural polymers are also biodegradable.

The time course of degradation should be planned and controlled. A degradable scaffold must support cell growth and provide the appropriate mechanical properties until the tissue is capable of fulfilling these functions. In the vasculature, a human artery typically experiences an average pressure of at least 100 mmHg (Guyton and Hall, 1996a). A material implanted to promote vessel regeneration must therefore supply the necessary strength and compliance until the native smooth muscle cells and fibroblasts are able to support the load. After the tissue is self-sufficient, it is optimal for the implanted material to completely degrade. The degradation timing can be controlled by adjusting polymer properties, such as the type and location of the degradable bonds. New methods of modifying polyanhydride networks have allowed for controlled variation of porosity, rate of degradation, and interactions with cells (Burkoth et al., 2000).

One must also consider the fact that the rate of tissue regeneration and polymer degradation will vary among individuals. The environment will affect the degradation rate as well. It has been shown that events such as fluid flow around a degradable PLGA scaffolds will decrease the degradation rate (Agrawal et al., 2000). A novel approach to bioresorbable scaffolds involves enzyme-dependent degradation, currently under development by Hubbell and coworkers (Schense et al., 2000; Ye et al., 2000). Rather than relying on the chemistry of the environment to degrade the material, this approach depends on the cells to control degradation. Until the cells enzymatically trigger degradation, the scaffold retains its mechanical and structural properties. This promising method has...
the potential to allow for individualized degradation that is completely dependent on the tissue-regeneration process.

Polymer degradation usually produces small degradation products. Some materials may degrade into small toxic by-products, even though the original material as a whole is nontoxic. When PLGA degrades into lactic acid and glycolic acid, the local pH may drop if the area is not well perfused (Wake et al., 1998). Also, if the by-products are immunogenic, the immune system may attack the area of desired tissue regeneration, and successful regeneration will not occur. To ensure biocompatibility, the toxicity of the degradation products ought to be evaluated.

16.3 BIOCHEMICAL COMPONENT

Once a material has been optimized based on its structural properties, bioactive agents can be incorporated to mimic normal regenerative environments found in vivo and further enhance tissue regeneration. These bioactive agents could consist of peptides, proteins, chemicals, oligonucleotides, genes, or living cells and can be included by two methods: (1) immobilization to the material and (2) release as diffusible agents. A combination of these two approaches could result in a diffusible signal that is chemoattractive and an immobilized signal that encourages cell attachment and proliferation. Table 16.4 provides a sampling of biochemical components incorporated into materials to enhance tissue growth.

16.3.1 Presentation of Immobilized Signals on the Scaffold

A scaffold material can provide cues that will direct and control the cell-matrix interactions to promote and regulate tissue regeneration. Biochemical signals such as matrix proteins, adhesive peptides, and growth factors can be incorporated into a material by nonspecific adsorption or through covalent immobilization. Since many mammalian cells require adhesion for cell viability, these molecules can provide the essential cues for cell attachment.

Matrix Proteins for Cell Adhesion. Cell adhesion in a receptor-mediated fashion is key for cellular processes such as intracellular signaling, synthesis of ECM proteins, and mitosis. Specific cell adhesion to scaffolds often occurs via ECM molecules that are presented on the scaffold surface. One adhesion study confirmed that smooth muscle cells adhere via integrins to extracellular matrix proteins (Nikolovski and Mooney, 2000). By immobilizing matrix proteins to the material, cell adhesion can be facilitated.

The ECM protein collagen includes the Arg-Gly-Asp (RGD) amino acid sequence that can facilitate specific interactions with cells via integrin receptors (Culp et al., 1997). Collagen has been covalently immobilized on PLGA surfaces to increase cell attachment (Zheng et al., 1998) and on polycarbonate and polyester membranes to enhance corneal epithelial tissue migration (Steele et al., 2000). A porous collagen membrane placed around a titanium implant resulted in increased bone repair in a model of alveolar ridge deficiency as compared with uncoated titanium implants (Zhang et al., 1999b). Collagen has also been used in combination with other ECM components to improve a polypropylene mesh for hernia repair. A collagen-glycosaminoglycan (GAG) matrix was placed around the mesh and shown to facilitate tissue growth and vascularization (Butler et al., 2001). The numerous applications for abundant ECM proteins such as collagen illustrate the usefulness of matrix proteins in tissue regeneration.

Laminin, another ECM protein, has also been used to modify materials, particularly for neural applications. When laminin was covalently immobilized on 3D agarose hydrogels, neurite extension from embryonic chick DRGs was stimulated. Further, immobilization was necessary, since laminin simply mixed into the agarose gel was unable to stimulate neurite extension (Yu et al., 1999). Uniform laminin substrates have also been used for the attachment of Schwann cells, but no preferential cell orientation was shown. When micropatterns of laminin and albumin were fabricated
TABLE 16.4 Examples of Biochemical Components Incorporated into Biomaterials for Tissue Regeneration

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on glass coverslips, Schwann cells attached only on the laminin surfaces and were oriented with the laminin patterns (Thompson and Buettner, 2001). These studies demonstrate how a more specialized protein such as laminin can also be used to enhance and direct tissue outgrowth.

Protein adsorption studies have demonstrated the importance of surface protein conformation. For effective interactions between cells and ECM proteins, the active domains of the proteins should be available for cell recognition. However, when a protein is adsorbed onto a surface to promote cell adhesion, the adhesion sequences will not necessarily be available. Changes in fibronectin conformation due to adsorption to glass and silane surfaces decreased endothelial cell adhesion strength and spreading (Iuliano et al., 1993). On the contrary, adsorption of vitronectin onto nanophase alumina resulted in a conformational change of the protein that increased the adhesion of rat osteoblasts, perhaps due to an increased exposure of the adhesive epitopes (Webster et al., 2001). Thus conformational changes should be considered when designing immobilization strategies.

Cellular attachment and proliferation are not the only parameters of regeneration to evaluate when optimizing tissue regeneration. It has been shown that increased cell adhesion due to adhesion-promoting surface modifications can result in a decrease of ECM production (Mann et al., 1999). When a biodegradable biomaterial is used, the cells must be self-sufficient when the material is gone, and this includes making their own ECM. A biomaterial with strong adhesive properties may be detrimental to overall tissue function. This example illustrates the importance of balancing cellular activities, such as cell adhesion and matrix production.

Adhesive Peptides. Immobilizing adhesive peptides to the material can also encourage cell attachment to biomaterials. Protein adsorption can result in a loss of quaternary and tertiary structure, but peptides should not denature or change conformation when immobilized to a surface. The most commonly used amino acid sequence for cell adhesion is the RGD sequence found in a variety of ECM proteins, including collagen, fibronectin, and vitronectin. Other peptide sequences used include Tyr-Ile-Gly-Ser-Arg (YIGSR) and Ile-Lys-Val-Ala-Val (IKVAV) from laminin, as well as Arg-Glu-Asp-Val (REDV) from fibronectin. All these sequences are short, so they are often contained within a slightly larger peptide to facilitate immobilization on scaffolds as well as interactions with cells.

RGD peptides work via interactions with cell surface integrin receptors, as shown by the receptor-mediated attachment of human umbilical vein endothelial cells (HUVECs) to silica substrates modified with RGD peptides (Porte-Durrieu et al., 1999). When immobilized on a surface, the RGD peptides typically localized in clusters rather than distributing uniformly (Kouvroukoglou et al., 2000). Clustering is advantageous since it has been shown to decrease the average peptide density required to support migration (Maheshwari et al., 2000). Moreover, the peptide surface density should be at or below the saturation level of the cellular receptors because peptide densities beyond that level have shown no further increase in cell attachment, spreading, or matrix production (Rezania and Healy, 2000). Consequently, there is an optimum surface density for RGD peptides that depends on the application.

Peptides are often used to enhance specific cellular activity with the materials. RGD peptides were covalently linked to quartz surfaces to examine their effect on rat calvaria osteoblast-like cell (RCO) adhesion. Although RCOs adhered and spread on both modified and unmodified quartz in the presence of serum proteins, the RGD-modified surfaces stimulated an increase in mineralization from the RCOs (Rezania and Healy, 1999). The RGD peptide also has been coupled to alginate hydrogels. Since these hydrogels are highly hydrophilic, they have minimal interaction with cells. C2C12 mouse skeletal myoblasts were cultured on 2D membranes of alginate with and without RGD-containing peptides. Only with the RGD peptides present did the myoblasts attach and spread (Rowley et al., 1999). Other studies have immobilized RGD peptides to a hyaluronic matrix to accelerate wound healing and regeneration in mice (Cooper et al., 1996) and skin healing and regeneration for pediatric burn patients (Hansbrough et al., 1995). Immobilizing RGD to polystyrene via Pluronic F108 allowed for increased fibroblast adhesion and spreading (Neff et al., 1998). In addition, bovine pulmonary artery endothelial cells were shown to have an increased migration on glass surfaces modified with either RGD or YIGSR containing peptides (Kouvroukoglou et al., 2000). The versatility of RGD peptides demonstrated by these studies suggests that RGD peptides can play an influential role in enhancing tissue regeneration.
Neurite extension can be controlled using immobilized peptides. RGD peptides linked to PLLAPEG-biotin surfaces via a streptavidin linker were shown to spatially control the adhesion and the direction of neurite extension from PC 12 cells (Patel et al., 1998). Neurite extension from PC 12 cells and NG108-15 neuroblastoma cells was also spatially controlled using laminin-derived peptides YIGSR and IKVAV bound to fluorinated ethylene propylene films (Ranieri et al., 1994). These studies demonstrate the potential impact of peptides on the regeneration of nerve tissues.

Another method of immobilizing peptides to a surface is to use self-assembled monolayers (SAMs) to modify a surface. Using the microcontact printing technique to create SAMs that both promoted (via RGD peptide) and inhibited (via PEG) cell adhesion, patterns of cell adhesion were established (Zhang et al., 1999a). A different self-assembly method used a molecule with a hydrophobic tail and a peptide head group that contains a collagenlike structural motif. These peptide-amphiphiles were able to promote the adhesion and spreading of human melanoma cells on tissue culture plates (Fields et al., 1998). Whether the immobilization technique involves SAMs or covalent chemistry, adhesive peptides are useful for modifying biomaterials to induce cell adhesion.

**Growth Factor Presentation.** In addition to ECM-related molecules, growth factors have been coupled to materials to provide a biochemical stimulus. Vascular endothelial growth factor (VEGF) immobilized to fibrin matrices had a greater mitogenic effect on human endothelial cells in vitro than soluble VEGF (Zisch et al., 2001). Also, basic fibroblast growth factor (bFGF) immobilized onto gas-plasma-modified polystyrene accelerated the formation of a confluent HUVEC layer in vitro, whereas soluble bFGF in culture medium was ineffective (Bos et al., 1999). Studies such as these indicate that immobilized growth factors can sometimes provide a more potent stimulation than soluble growth factors. In another study, TGF-β1 was covalently immobilized to PEG hydrogels and found to increase the matrix production of vascular smooth muscle cells (Mann et al., 2001). Peptides based on the active domains of growth factors have also been utilized as biochemical cues. Alginate hydrogels have been covalently modified with a 20-amino-acid sequence derived from bone morphogenic protein-2 (BMP-2) to induce bone formation in vivo. After 8 weeks, the gel was vascularized, and osteoblasts had formed new trabecular bone within the alginate pores (Suzuki et al., 2000). Therefore, by immobilizing growth factors to biomaterials, a range of cell responses can be achieved.

### 16.3.2 Presentation of Chemoattractive, Diffusible Signals on the Scaffold

In addition to immobilized signals, the body also creates chemoattractive gradients using diffusible factors to guide cell migration. To imitate this approach, a bioactive agent can be incorporated into the biomaterial to diffuse away and provide a biochemical cue. For agent release, the biomaterial could consist of one component that fulfills both the role of the scaffold to support the cells and the role of the delivery system to release the biochemicals. Alternatively, a two-component system using one material for the scaffold and a different material for the delivery system could be employed.

**Scaffold-Drug Delivery: One-Component System.** Using the biomaterial scaffold as a drug-delivery device is a simple means of incorporating a diffusible agent into the material. A bioactive agent incorporated into the biomaterial will slowly diffuse away after implantation to create a concentration gradient. Diffusible agents have been included in matrices by adding them either during or after the fabrication process.

Biodegradable polyesters are among the materials usually loaded by adding the biochemicals during fabrication. Acidic fibroblast growth factor (aFGF) was incorporated into PDLLA-PCLA copolymers for nerve regeneration (Maquet et al., 2001), and tetracycline was added to a PLLA film/PGA mesh to stimulate bone regeneration and marrow formation in a rat craniotomy defect (Park et al., 2000). PLLA and PLLA-TCP membranes designed to release platelet-derived growth factor-BB (PDGF-BB) enhanced bone formation and allowed for complete bony reunion in a critical-sized defect in rats (Lee et al., 2001; Park et al., 1998). Also, titanium coated with PDLLA that contained...
insulin-like growth factor and TGF-β1 significantly accelerated fracture healing in rodents as compared with implants without growth factors (Schmidmaier et al., 2001). Fabricating scaffolds in the presence of bioactive agents is an effective method to include diffusible factors in biomaterials.

Another method for loading a biomaterial is to add the bioactive agent after the matrix has already been formed, such as by soaking a matrix in the agent solution. This method is frequently utilized for loading collagen scaffolds. An absorbable, macroporous collagen sponge soaked in a recombinant human bone morphogenic protein (BMP) solution increased the osteoinductive activity of the material (Uludag et al., 1999). To promote cartilage formation, collagen sponges were soaked in bFGF. They induced tissue regeneration at 1 week and mature chondrocytes at 4 weeks postimplantation, whereas sponges without bFGF did not show significant cartilage regeneration (Fujisato et al., 1996). These studies suggest that growth factor release can be a potent stimulator of tissue regeneration.

The release of angiogenic factors to encourage neovascularization allows for increased tissue survival. Methods to support this facet of tissue regeneration have included the slow release of angiogenic factors such as VEGF (Eiselt et al., 1998). PLGA foam scaffolds fabricated to release VEGF have been shown to increase the proliferation of human dermal microvascular endothelial cells as compared with scaffolds without VEGF (Murphy et al., 2000). Thus various aspects of regeneration can be enhanced by appropriately selecting the material and the bioactive agent to be released.

**Scaffold-Drug Delivery: Two-Component System.** An assortment of drug-delivery systems is available and can be incorporated into a scaffold for tissue regeneration. This two-component method of delivering agents is particularly useful for biomaterials that cannot be loaded with agents by the previously described methods or are not capable of providing adequate slow release. One designated delivery system is the PLGA microsphere system (Fig. 16.4). As loaded microspheres degrade, they release the particles trapped within and provide the diffusible agent. PLGA microspheres loaded with heparin, known for its antiproliferative and anticoagulative activity, were embedded in alginate films. These films were then wrapped around vein grafts and denuded carotid arteries in rats and shown to reduce intimal hyperplasia as compared with control films without heparin (Edelman et al., 2000). PLGA microspheres have also been loaded with VEGF to induce the proliferation of human dermal microvascular endothelial cells (Eiselt et al., 1998). To promote angiogenesis, gelatin microspheres were loaded with bFGF and incorporated into an artificial dermis consisting of an outer silicone layer and an inner collagen sponge layer. Implants with the bFGF-loaded microspheres were shown to accelerate fibroblast proliferation and capillary formation in vivo (Kawai et al., 2000). Another delivery system used to provide chemical gradients is the lipid microtubule delivery system (Meilander et al., 2001). Lipid microtubules are hollow tubules that can be loaded with a bioactive agent and embedded into the biomaterial. Due to the concentration gradient between the tubules and the external environment, the agent is released. Lipid microtubules loaded with nerve growth factor (NGF) and incorporated into agarose hydrogels have been shown to directionally stimulate neurite extension from 3D cultures of chick embryonic DRGs (Yu et al., 1999), as depicted in Fig. 16.5. These studies demonstrate that a specialized delivery system is an effective means to provide a diffusible biochemical signal within a scaffold.

**Gene-Delivery Scaffolds.** Gene therapy can also provide a biochemical component to promote tissue regeneration. Instead of releasing a protein, the gene for that protein can be released to transduce cells for local protein synthesis. This is especially useful for sensitive proteins with short in vivo half-lives. One application that has benefited from gene delivery is wound healing. Gas-foamed PLGA matrices loaded with plasmid DNA encoding PDGF-B increased the formation of granulation tissue and vascularization following subcutaneous implantation in rodents (Shea et al., 1999). Likewise, collagen matrices containing adenoviral or plasmid DNA for PDGF-A or PDGF-B increased granulation tissue, reepithelialization, and neovascularization to result in accelerated wound healing (Chandler et al., 2000).

Gene delivery has also been used to enhance bone regeneration in vivo. An adenoavirus encoding bone morphogenic protein-7 (BMP-7) was mixed with a collagen carrier and implanted in mice to
produce ectopic formation of cortical and trabecular bone with a marrow cavity after 4 weeks (Franceschi et al., 2000). Similarly, plasmid DNA encoding a fragment of parathyroid hormone (PTH) was incorporated in a collagen gel to reproducibly promote bone regeneration in a canine model of bone injury. Since the DNA was shown to be incapable of diffusing out of the initial form of the gel, the DNA remained localized at the injury site until cells arrived and degraded the collagen

![FIGURE 16.4 Scanning electron microscope image of PLGA microspheres of various sizes. After microsphere fabrication, sieves can be used to select a specific size range (bar = 10 µm). (Courtesy of J. Gao, Ph.D.)](image)

![FIGURE 16.5 Schematic of directional neurite extension from chick DRGs in a 3D agarose culture. NGF concentration gradients were established by the microtubule delivery system, and neurite extension was directed preferentially toward the NGF-loaded microtubules (diagram not to scale).](image)
matrix (Bonadio et al., 1999). To test the effects of using two plasmids simultaneously, collagen sponges were soaked in a solution of plasmid DNA encoding either the osteoinductive protein bone morphogenic protein-4 (BMP-4), a fragment of PTH, or both BMP-4 and the PTH fragment. The loaded sponges were implanted in critical defects in the femoral diaphysis of adult rats. Defects with either the BMP-4 or PTH fragment plasmid healed completely and demonstrated the same mechanical strength as the unoperated femur. Moreover, combination gene therapy, with a collagen sponge containing plasmid DNA encoding both BMP-4 and the PTH fragment, was found to further enhance bone regeneration (Fang et al., 1996). As these examples illustrate, gene therapy is a useful method to stimulate tissue regeneration, especially with the use of multiple genes. Advances in the field of gene therapy will extend the use of this promising technique to a variety of tissue-regeneration applications.

16.3.3 Presentation of a Living Component

A more recent approach to enhance the regenerative effort incorporates cells into tissue-engineered biomaterials. Rather than relying entirely on the migration of host cells into the implant, cells can be seeded in the biomaterial. Differentiated cells, as well as stem cells, are being used for regenerative therapies. In addition, cells can be used in combination with other biochemical therapies to further enhance regeneration.

Cell Seeding. The seeding density of cells on the implanted material can be adjusted for each application. It was shown that the final cell concentration of smooth muscle cells on PGA fiber matrices was proportional to the seeding density (Kim et al., 1998). Likewise, the osteogenic activity of stromal osteoblasts cultured on 3D PLGA foams was found to depend on the seeding density of the cells (Ishaug et al., 1997). An appropriate seeding density can be chosen based on the rate of implanted cell proliferation and host tissue proliferation. An implant with a low density of cells allows for the proliferation and migration of both implanted and host cells. If the cellular density on the implant is similar to that of the tissue in vivo, minimal proliferation of the host tissue is necessary. This could be useful in tissues, such as central nervous system tissue, that only have minimal regenerative capacity. In addition, the seeding method affects the cell distribution. Dynamic seeding methods result in a larger number of adherent cells as well as a more uniform distribution of the cells (Kim et al., 1998). Accordingly, the effective use of cells to stimulate tissue regeneration will involve choosing an appropriate cell seeding density and method.

Differentiated Cell-Scaffold Systems. A number of differentiated cell types have been used to aid in the regeneration of tissue in vivo. Endothelial cells are often included for vascular tissue regeneration or for neovascularization of scaffolds. Endothelial cells were perivascularly transplanted in Gelfoam matrices around balloon-denuded rat carotid arteries to control the tissue healing response and reduce intimal hyperplasia (Nathan et al., 1995). Endothelial cells have also been used in the sodding of expanded polytetrafluoroethylene (ePTFE) vascular grafts to enhance the regeneration of an endothelial cell lining in a rat aortic graft model (Ahlswede and Williams, 1994). Endothelial cells and vascular myofibroblasts were seeded onto polyglycolic-acid–poly-4-hydroxybutyrate copolymers to create small-diameter vascular grafts (Hoerstrup et al., 2001). To improve scaffold vascularization, endothelial cells and dermal fibroblasts were added to a skin equivalent consisting of a chitosan-linked collagen-GAG sponge containing keratinocytes. The endothelial cells and fibroblasts promoted the formation of a network of capillarylike structures in vitro and may serve as a framework to facilitate vascularization upon implantation (Black et al., 1998). Other studies have incorporated fibroblasts on spongy collagen to aid in the healing of skin defects (Kuroyanagi et al., 2001), chondrocytes in fibrin glues to result in actively proliferating and ECM-producing cells (Sims et al., 1998), keratinocytes on collagen-coated dextran microcarriers to reconstitute epithelium (Voigt et al., 1999), and hepatocytes on poly anhydrides, polyorthoesters, or PLGA fibers for possible liver regeneration (Vacanti et al., 1988). These studies suggest that numerous cells types have the potential to provide a living component in biomaterials.
**Scaffold Prevascularization.** Vascularization is important in implants seeded with cells because these cells will immediately require a source of nourishment, most likely via the bloodstream. One approach to ensure an adequate vascular supply when cells are implanted is to implant a cell-free scaffold to allow for fibrovascular tissue ingrowth prior to seeding the cells. Using a PVA foam, the timing of in vivo fibrovascular tissue formation was determined. It was concluded that cells should be seeded into the material as soon as the fibrovascular tissue has reached the center of the implant (Wake et al., 1995). A similar method implanted an encapsulation chamber prior to adding the cells with the intent of increasing vasculature around the chamber, since normal wound healing results in vessel regression during the second week. Cellulose membranes with 8-µm pores were able to maintain the vascularization of the fibrous capsule that surrounded the membrane beyond 2 weeks, allowing for a sustained vascular supply (Padera and Colton, 1996). Such methods may provide improved local vascularization and mass transport at the time of cell implantation. However, these strategies may have limited application because they require two implantation procedures, one for the scaffold and one for the cells.

**Cell-Scaffold Constructs for Neuroregeneration.** Neurodegenerative diseases are prime candidates for cell therapy approaches to tissue regeneration. One disease that has received considerable attention for cell therapy approaches is Parkinson’s disease. In one study, rat adrenal chromaffin cells (ACC) were seeded on collagen-coated dextran or glass beads and injected into the brains of hemiparkinsonian rats. These animals showed significant behavioral recovery over the 12-month study. Animals receiving only the ACC without the microcarriers showed improvement at 1 month but reverted to the original diseased state by 2 months, demonstrating that the scaffold was necessary for prolonged treatment and recovery (Borlongan et al., 1998; Cherksey et al., 1996). Bovine chromaffin cells have also been used to treat hemiparkinsonian rats. Using alginate-polylysine-alginate as a microencapsulator, the cells were implanted in hemiparkinsonian rats and shown to decrease apomorphine-induced rotation (Xue et al., 2001). Likewise, human and rat fetal ventral mesencephalon cells have also been seeded onto collagen-coated dextran microcarriers as a possible treatment for Parkinson’s disease. The cells had an increased survival when attached to the microcarriers versus cell suspensions (Saporta et al., 1997). To supply dopamine, PC 12 cells capable of secreting dopamine were encapsulated in agarose–poly(styrene sulfonic acid) and grafted into the corpus striatum of guinea pigs. The cells were stained with a tyrosine hydroxylase antibody, suggesting that they continued to secrete dopamine in the brain. In addition, there was no immunological rejection or tumor formation when the biomaterial was used (Date et al., 1996). Cell therapy also has potential to treat Alzheimer’s disease. In one such effort, baby hamster kidney cells were genetically engineered to overexpress NGF. They were then mixed with vitrogen and infused into poly(acrylonitrile-co-vinyl chloride) copolymers (PAN/PVC) to reduce the degeneration of basal forebrain cholinergic neurons (Emerich et al., 1994). These findings regarding Parkinson’s disease and Alzheimer’s disease suggest that biomaterials, either as a scaffold or as a means of encapsulation, play an important role when using cells as a treatment for neurodegenerative diseases.

**Combination Therapy.** In vivo, cells interpret and react to numerous signals simultaneously. Taking advantage of this ability, multiple biochemical cues can be provided for tissue regeneration. One study evaluated human skin fibroblasts grown on fibrin microbeads. The microbeads with cells were shown to enhance wound healing as compared with fibroblasts or microbeads by themselves. However, when the diffusible growth factor PDGF-BB was included with the fibroblast-fibrin microbeads, the healing response was further improved (Gorodetsky et al., 1999). Growth factors that encourage neovascularization can also be included with the cells. In one such approach, murine myoblasts were cultured in 3D scaffolds of Matrigel containing either bFGF or hepatocyte growth factor to promote angiogenesis. When the scaffolds were implanted in a model of ectopic muscle regeneration, the presence of the growth factors increased angiogenesis, resulting in improved cell viability and enhanced myogenesis (Barbero et al., 2001).

Gene therapy and cell therapy have been combined to result in murine stromal cells transduced to produce BMP-2. The cells were seeded on a PLGA-HA matrix and found to induce heterotopic bone regeneration (Laurencin et al., 2001). Likewise, rat dermal cells were infected with a retrovirus
containing the PDGF-B gene and seeded onto PGA scaffolds to modulate wound healing (Breitbart et al., 1999). In another study, fibroblasts engineered to express neurotrophic factors were seeded onto poly-N-(2-hydroxypropyl)-methacrylamide hydrogels with RGD-containing peptides. This therapy, incorporating cells, DNA, and adhesive peptides into one material, was shown to enhance optic nerve regeneration. In addition, when the cells produced two neurotrophic factors, axonal growth into the hydrogels was greater than with either growth factor alone (Loh et al., 2001). These examples illustrate the positive regenerative effects when a biomaterial is designed to utilize several parameters in concert.

**Stem Cell-Scaffold Systems.** Stem cells, also known as progenitor cells, are pluripotent cells with the ability to differentiate into a variety of cell types. The most widely studied stem cell is the mesenchymal stem cell (MSC) derived from bone marrow. In the embryo, these cells give rise to skeletal tissues, including bone, cartilage, tendon, ligament, marrow stroma, adipocytes, dermis, muscle, and connective tissue (Caplan, 1991).

Mesenchymal stem cells are relevant for tissue regeneration because of their ability to differentiate, a trait they share with many cells that facilitate tissue repair in vivo. Harnessing this capability by combining MSCs, biomaterials, and the appropriate cues for differentiation should allow for regeneration of the skeletal tissues just mentioned. A composite matrix of gelatin and esterified hyaluronic acid matrix seeded with MSCs enabled osteochondrogenic cell differentiation when implanted subcutaneously in mice (Angele et al., 1999). Similarly, MSCs were harvested from rabbit bone marrow and seeded on a hyaluronan-based 3D scaffold to fill an osteochondral defect in rabbits. While the plain scaffold was able to enhance osteochondral repair, the morphology of the repair improved when progenitor cells were included (Radice et al., 2000). For cartilage regeneration, MSCs were seeded onto collagen sponges and shown to augment meniscus cartilage regeneration in rabbits (Walsh et al., 1999). Another study demonstrated the effectiveness of MSCs for bone regeneration. MSCs were seeded onto coral scaffolds and implanted in a bone defect in sheep. The MSC scaffolds were more likely to develop into new cortical bone with a medullary canal for clinical union than the coral scaffold alone (Petite et al., 2000). MSCs have also been suspended in collagen gels and implanted into tendon defects to improve tendon regeneration and biomechanical properties (Awad et al., 1999; Young et al., 1998). These studies with MSCs are just beginning to realize the potential of stem cells as tools for tissue regeneration.

As a combination therapy, stem cells were used in conjunction with gene therapy to further enhance regeneration. Human bone marrow–derived mesenchymal stem cells were infected with an adenovirus containing the BMP-2 gene, cultured on a collagen matrix, and transplanted in murine radial defects. Unlike the sham-infected MSCs, BMP-2–infected MSCs were able to differentiate into chondrocytes in vivo, regenerate bone, and bridge the radial gap (Turgeman et al., 2001). In a similar model, genetically engineered pluripotent mesenchymal cells expressing BMP-2 were compared with a nonprogenitor cell line also genetically engineering to express BMP-2. The cells were seeded onto collagen sponges and transplanted into a bone defect in mice. Even though the nonprogenitor cells had a much higher BMP-2 expression in vitro, they showed no organized bone formation in vivo. The progenitor cells, despite a lower gene expression in vitro, induced orderly formation of bone and cartilage (Gazit et al., 1999). Again, materials designed with more than one factor to enhance regeneration proved superior to single-factor designs.

**Commercial Cell-Based Tissue-Engineered Products.** Tissue-engineered products containing cells are beginning to appear on the commercial market. Apligraf, a bilayered product developed and manufactured by Organogenesis (Canton, MA) and marketed by Novartis Pharmaceuticals Corporation (East Hanover, NJ), is composed of neonatal-derived dermal fibroblasts, keratinocytes, and bovine collagen. It has been approved for the treatment of venous ulcers in the United States (Eaglstein et al., 1999). Carticel, from Genzyme Tissue Repair (Cambridge, MA), consists of healthy autologous chondrocytes that are harvested, proliferated in vitro, and implanted into sites of injured cartilage to improve the tissue. Dermagraft (Advanced Tissue Sciences), a dermal substitute consisting of dermal fibroblasts on a resorbable substrate, has recently received Food and Drug Administration (FDA) approval in the United States. In addition, several other tissue-engineered products are
available in Europe and other countries but are not yet approved for marketing in the United States. These products include Epicel (Genzyme Tissue Repair, Cambridge, MA) and VivoDerm (ER Squibb and Sons, Inc., Princeton, NJ; ConvaTec, Ltd., authorized user).

16.4 CONCLUSIONS

Tissue regeneration allows for the development of self-renewing, responsive tissue that can remodel itself and its matrix. A number of methods to encourage tissue regeneration are currently available, most of which place the host tissue in the primary role and the biomaterial in a supportive role, mimicking permissive in vivo environments. Since a barrage of structural and biochemical cues orchestrates tissue regeneration in vivo, the design of biomaterials for tissue regeneration must progress to include a combination of design parameters. As knowledge of cell and molecular biology continues to advance, more parameter combinations will be discovered, and the effectiveness of biomaterials designed for tissue regeneration will undoubtedly improve and extend to a variety of organs.

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